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MAMMARY NEOPLASMS OF THE BITCH

By

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INTRODUCTION

If the cause of mammary tumours of the bitch is to be understood, it is first necessary to know something about their histogenesis and about the components of the mammary gland which are concerned in their development. In this paper, an attempt is made to throw further light on the possible inter-relationships of the neoplasms of the canine mammary gland—a group of tumours of a great variety of histological structure and sometimes of uncertain histogenesis. Particular attention is given to the possible histogenesis of the mucoid, cartilaginous and bony elements so often present in benign, and to a lesser extent in malignant, tumours of these glands.

From the beginning of 1950 until the end of July, 1956, a macroscopic and histological examination of mammary neoplasms from 424 dogs has been made.

METHODS AND MATERIAL

Sections from all tumours were stained by haematoxylin and eosin, and selected sections by van Gieson, Masson's trichrome (haematoxylin erythrosin-saffron or ponceau-fuchsin light green), Alcian blue, and P.A.S. Some tumours were examined histochemically for alkaline phosphatase activity.

The affected animals were all females: two had been castrated in adult life. One of the bitches was pregnant. The tumours from 381 bitches were removed surgically, while the others came from bitches of which 28 were examined post mortem at the College, and 15 were examined post mortem by practising veterinary surgeons.

RESULTS

Age incidence. The age, recorded for 318 bitches, ranged from 2 to 17 years (average 9 years). The number of affected animals of each age was as follows:

Age (years)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
No. of bitches	0	2	1	5	16	17	47	49	40	51
Age (years)	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
No. of bitches	20	38	17	11	0	3	1	0	0	0

Breed incidence. The breed was specified for 234 bitches, the more numerous being: Spaniel (all types) 62, Scottish Terrier 17, Fox Terrier 15, Poodle 13, Dachshund 12, Alsatian, Labrador, Retriever each 10. No other breed provided more than 8 cases. There were in addition 86 mongrel or cross-bred dogs.

Site of tumours. The majority of tumours were in one or other of the two hinder glands (numbers 4 and 5): of 160 tumours whose location was recorded, 105 occurred in these glands, as against 19 in the middle pair and 46 in one or other of the two anterior pairs.

Classification of the tumours. The tumours were first classified as benign or malignant. The malignant tumours comprised (i) those that proved themselves to be malignant by producing metastases; (ii) those that had a histological structure similar to that shown by these metastasizing tumours; (iii) those that otherwise were malignant by usual criteria, such as local invasive growth, numerous mitoses, cell anaplasia. The border between the benign and malignant groups was not clear-cut.

By these criteria, 186 of the 424 bitches bore malignant mammary tumours (accompanied in 11 cases by benign tumours), and 249 had benign mammary tumours. One of the 186 bitches had malignant mammary tumours of two different kinds.

BENIGN TUMOURS (249)

The benign tumours were of two main types, "simple" or "complex", according to whether they appeared to consist of a neoplastic growth of one, or of more than one, type of cell. The "complex" tumours included those lesions which have been classified by other writers as papillary cystadenomas, mixed tumours, or fibrocystic disease respectively.

Simple Benign Tumours (19)

There was a neoplastic growth of one kind of cell only, of either connective tissue or epithelial type.

Connective tissue type (11). These comprised 9 lipomas, and 2 fibromas.

Epithelial type (8). There were 8 simple adenomas. The tumours varied from $1\frac{1}{2} \times 1$ to 11×9 cm. on the cut surface, being generally rather large. They approached a spherical shape, with well-defined edges, and were not attached to the skin. The cut surface was white or greyish-white, faintly lobulated and without obvious cysts. The consistency varied.

Histologically, acini were well-developed except in three tumours, where small solid structures, resembling ductules but without lumens, predominated. The acini were lined by cuboidal or medium columnar cells and contained a pink-staining (H. & E.) colloid material. Some cells had clear cytoplasm and a double layer of epithelium was seen in some duct-like formations.

Complex Benign Tumours (230)

Although there was wide variation in their individual structure, the majority of the benign tumours (230 out of 249) showed sufficient gradations of appearance to allow of their inclusion in one group.

Their characteristic features were, firstly, that there was present a cellular component which was closely related to the true epithelial cells, and often played a role greater than that of the secretory epithelium; and, secondly, that the tumours showed a tendency to develop mucoid, cartilaginous or bony areas. The significant non-epithelial cells, which often lay between true epithelial cells and the basement membrane, or, where that was not apparent, the collagenous connective tissue, will be called for the present "interposed" cells, leaving their identity to be discussed later.

These tumours could be arranged in a series of increasing complexity of structure. The least complex were tumours of glandular structure without any relative preponderance of interposed cells. In the next more complex type, there were relatively increased numbers of interposed cells in proportion to the true epithelium. Yet more complex were tumours showing mucoid areas, generally in relation to proliferations of interposed cells. In still more complex tumours, cartilage, bone and even bone-marrow were present. All these components were embedded in fibrous tissue of varying amount.

One or more of the various components might predominate and the components seen in less complex tumours might be absent from more complex tumours. The relationship of bone to cartilage, or cartilage to mucoid tissue, and of mucoid tissue to interposed cells, suggested that these often represented stages of increasing differentiation of one general type of tumour. Of the 230 benign complex tumours 40 contained bone, an additional 63 contained cartilage but no bone, and a further 67 showed mucoid tissue, but no cartilage or bone.

Macroscopic Appearance of the Benign Complex Tumours

The macroscopic appearances were, in accord with the histological structure, very variable. The tumours ranged in size up to 10 cm. or more across. They lay subcutaneously, sometimes within a teat duct, and were not often ulcerated, unless they were large or bony. They varied in consistency and in shape, and were accompanied in some cases by obvious local mammary glandular hyperplasia.

The tumours were formed of single nodules, or of a few or many nodular masses more or less closely aggregated (Fig. 11). The edge of the tumours was sometimes well-defined, but at other times was made out with difficulty from the local hyperplastic tissue. The tumours were sometimes fairly homogeneous and solid, white or grey, firm masses, with more or less obvious cartilage or bone, or they showed fine or coarse septa of fibrous tissue with variable browner softer tissue intermingled. There was obvious cyst formation in some tumours, single cysts sometimes forming nearly the whole tumour. The cysts contained clear or opaque fluid, which was colourless or, more often, brown in colour. Papillary projections

often grew into the cyst lumens. Some of the tumours were bone-hard, heavy, oval or spherical bosselated masses, in which a radiating structure of cancellous bone, with variable peripheral caps of cartilage, might be seen on the cut surface.

Histology of Benign Complex Tumours

The complex tumours were often accompanied by lobular hyperplasia of local glandular tissue and, in their simplest and presumably earliest forms, the neoplastic areas were just becoming distinct from the local hyperplastic lobules. One, a few or many lobules of the mammary gland acquired an adenomatous appearance, with variable cyst development, and often there were intracystic papillary growths. The neoplasms arose from and comprised ductal as well as acinar structures, the former predominating in papillary cystadenomas.

The different lobular areas tended to have similar appearances, although some might show a greater degree of complexity. The epithelial and the interposed cells were supported by connective tissue of variable, and sometimes predominant, amount which usually contained numbers of plasma cells and other mononuclear cells, including mast cells, and cells containing brown pigment granules. The fibrous tissue was in some cases oedematous, or hyaline, and often showed degrees of sclerosis, which was most marked around cystic duct-like structures or in the centre of lobular growths.

In these tumours, the interposed cells sometimes formed a prominent single layer around cystic structures. In papillary cystadenomas, they could be seen to turn in at right angles to the limiting membrane to form the cores of small papillary projections, and they were usually prominent in the cores of larger papillae where they often resembled smooth muscle cells. The intracystic papillae were much branched and some were multi-radiculate.

Occasionally, scattered patches of squamous metaplasia of the lumenal epithelium were present, with variable keratinization. Very occasionally, there was sebaceous metaplasia. The periacinar and periductal basement membranes showed variable hyaline changes and might entrap numbers of interposed cells. Sometimes the basement membranes were frayed out, and on rare occasions were calcified. The brown pigment-bearing cells of the stroma appeared to pass through the walls of the acinar or cystic structures, where they could be seen just inside the basement membrane, in the lumenal epithelial layer or in the lumen contents. Occasional drops of mucin and a variable number of "cholesterol clefts" were seen in these contents, which stained bright red with eosin, and were P.A.S. positive.

In the next stage of complexity, there were one, a few or many foci of increase of the interposed cells. They lay in the walls of ducts or of cysts, or in the cores of papillae, or in the walls of acinar

structures. They were sometimes, but not always, clearly intra-acinar, that is, within the limits of the basement membrane. Some tumours showed relatively so many of these proliferated interposed cells that the true lumenal epithelial component seemed, by comparison, very scanty or even absent—its location was often indicated by the red-stained lumen contents. The interposed cells sometimes formed bundles resembling smooth muscle. Amongst the interposed cells were some with dark, narrow nuclei considered to be fibroblasts of intralobular connective tissue origin.

The proliferated cells tended to form a loose meshwork of individual cells, or occasionally cords or bundles or rows of cells, or an apparent syncitium. They had pale, oval nuclei without prominent nucleoli. They might have a stellate shape and often appeared to be lying in almost unstained fluid. Some of the interposed cells were large and plump, with red-staining cytoplasm—they might then have a distinctly epithelial aspect. There was often a marked ambiguity of location of the proliferating cells, so that they were neither clearly interstitial nor clearly intra-acinar. This may indicate a true variability of position of cells of the same interposed kind.

A further stage in the development of these complex tumours was the appearance of mucin. This generally appeared in tumours in which an interposed cellular component was present, and was nearly always in relation to cells of this kind. The mucin, which was P.A.S. negative and stained with Alcian blue, appeared in H. & E. sections as blue-staining material of varying intensity, which lay chiefly between interposed cells. The latter sometimes had a P.A.S. positive cytoplasm. Some mucin appeared to be in the cytoplasm of interposed cells, which gave the appearance of bursting at their edges to leave a mesh of cytoplasm with scattered nuclei and without definite cell outlines. Sometimes the mucin appeared as a faint, lace-like material, progressing to a deep blue-staining, and often vacuolated, substance. The cells embedded in the mucin were often stellate, but sometimes had no obvious cytoplasmic processes. The proportion of cells to mucin was variable. The mucin in part appeared to soak into thickened, hyalinizing basement membranes which were broken up in the groups of interposed cells. There was an ambiguity of position of some of the mucoid cell groups corresponding to that of interposed cells. The mucoid foci varied in number, in size and in intensity of staining. Occasionally, however, the presence of multiple, small, more or less equal-sized foci of mucoid tissue suggested the effect of some agent acting at about the same strength and at about the same time on a similarly-susceptible cellular tissue: this would be in accord with a reaction to a hormonal stimulus.

In the still more complex tumours, cartilage was present. This varied from a very imperfect to a well-defined cartilage with prominent chondrin balls. In some tumours, a similar ambiguity of position was noted as for the mucoid and interposed-cell areas,

but in others the cartilage was in continuity with mucoid cellular tissue of which it seemed to be a development: the cells in the cartilage matrix were then continuous with those in the mucoid matrix and were taken to be interposed cells.

In the development of the cartilage matrix, there was partly a change in the mucoid material which did not now stain with Alcian blue, and appeared denser in H. & E. preparations with a greater degree of basophil staining around the enclosed cells, and partly an incorporation of basement membranes which showed hyaline thickening. There was often no definite perichondrium around the cartilage (Fig. 1), and mucoid tissue of interposed cell origin could sometimes be found at the edge of the cartilage masses (Fig. 2), even when they appeared to be definitely interstitial in position. However, an origin by metaplasia of intralobular interstitial connective tissue was strongly suggested in some instances. Even in some ostensibly interstitial cartilage foci, there were seen to be embedded epithelial structures (Fig. 3). It is concluded that some of the cartilage in these tumours was a development of mucoid tissue of interposed cell origin, and some was of metaplastic connective tissue origin. In one or two cartilage foci, vascularization presaged endochondral ossification.

In the most complex benign tumours, bone, and even bone marrow, was developed, but it was not possible to say that the bone was produced by the same cell as the cartilage. In a few instances, osteoid tissue seemed to be formed through a hyaline change in the basement membrane or of the intralobular connective tissue. In other instances, osteoid and chondroid tissue merged in trabeculae of mixed structure: this may possibly indicate that the cartilage and bone were formed by the same kind of cell, and thus either by metaplasia of connective tissue or by interposed cells. However, the most usual appearance was that the bone was formed by osteoblasts, by processes of endochondral or of membranous ossification.

The bone matrix was sometimes laid down in irregular plates on the surface of cartilaginous masses, but endochondral ossification was present in places with erosion of cartilage masses in which a linear arrangement of cartilage cells might simulate epiphyseal plates (Fig. 4). Osteoclasts were often prominent and lines of apposition in the bone masses suggested that more than one generation of bone matrix was present. Some of the tumours consisted chiefly of bone and cartilage, and in larger tumours there was a marked radiating arrangement of bony and cartilaginous trabeculae with cartilage caps at the periphery of cancellous bone.

The cartilage undergoing ossification was in some cases related to mucoid areas of interposed-cell type, while in others it appeared to be interstitial. The bone sometimes lay in the convexities of branching duct-like systems, or the epithelial structures might form branching cores in the interstices of bony and cartilaginous trabeculae. Some acinar formations were embedded in bone

matrix. In the cancellous bone, bone marrow of fatty or myeloid appearance was present (Fig. 5).

The intramembranous bone was embedded in fibrous tissue, which often contained spindle-shaped cells resembling interposed cells.

Nature of the interposed cells. The interposed cells are considered to be myoepithelial or, in the sense of the term used by Lennox, Pearse and Richards, (1952), myoepitheliomatous cells, that is, they represent the basket cells of the acini, or the corresponding cells lying just inside the basement membrane of the ducts. In favour of this identification are their position, their morphology, their staining reactions and their alkaline phosphatase activity.

(i) The characteristic position of the interposed cells is that of the myoepithelial cells: that is, external to the lumenal epithelium and inside the basement membrane, or the limiting layer of intralobular connective tissue. No other cells are at present known to occur normally in such a position and, further, continuity of the interposed cells with undoubted myoepithelial cells can be demonstrated.

(ii) The shape of the interposed cells, myoid or stellate, is that of myoepithelium.

(iii) Some interposed cells stain red with Masson's trichrome, as does normal myoepithelium. This staining reaction was, however, only present in 5 of the 25 tumours tested and was never so strong as for normal myoepithelium.

(iv) Because of the inconstant results of Masson's trichrome stains, it was decided to make use of the fact that, histochemically, normal myoepithelium shows alkaline phosphatase activity (Dempsey, Bunting and Wislocki, 1947; Silver, 1954; Marrable, unpublished) to try and identify the interposed cells. So far, 31 tumours (22 benign, 9 malignant) have been tested for alkaline phosphatase activity: in the first 6 instances, by the Gömöri method (Pearse, 1953), in the next 7 supplementing this by the modified coupling azo dye method for paraffin sections, also described by Pearse, using his salt 9 (5-chloro-o-toluidine), and in the succeeding 18 instances by the azo dye method alone.

Appropriate reference will be made to the results with the malignant tumours later. Of the benign tumours 20 were complex. In these, cells having the character of interposed cells in ordinary haematoxylin and eosin sections were either positive or negative when tested for alkaline phosphatase activity: both positive and negative cells could be present in the same section.

Of those interposed cells that were negative, some were apparently interstitial in position—these could be unrelated to positive interposed or myoepithelial cells, or be in continuity with them; others were in the position of myoepithelium, which they appeared to replace (Fig. 6). The positive cells were either in the position of myoepithelium, or continuous with myoepithelial cells (Fig. 7),

and they might in turn be in continuity with some of the negative cells (Fig. 8). The positive or the negative cells might form bundles in the cores of papillae or in the stroma, or lie in the pools of mucin (Figs. 9 and 10).

Interposed cells which showed alkaline phosphatase activity were demonstrable in 15 of the 20 complex tumours. They were positive after varying periods of incubation—even after 5 minutes.

In two tumours containing bone, the osteoblasts near bone were positive after 5 minutes, as were the normal myoepithelial cells, and in one tumour with cartilage, the perichondrium showed activity in sections incubated for 5 minutes. In two tumours, capillary walls were positive and, in one, so was what appeared to be interstitial connective tissue, but in general, the only cells reacting strongly and early were myoepithelial or interposed cells. The observations support the view that the interposed cells are of myoepithelial nature.

MALIGNANT TUMOURS (187)

Of the mammary tumours in this series, 187, from 186 bitches, were classified as malignant. Of these, 87 were of epithelial structure and classed as carcinomas, and 73 were of non-epithelial structure and classed as sarcomas. The other 27 had characters of both these types and will be described separately as complex malignant tumours.

Definite proof of malignancy, the occurrence of metastasis, was present in 41 bitches bearing malignant tumours—21 with carcinomas, 13 with sarcomas and 7 with complex malignant tumours. There was thus unequivocal proof of malignancy in only 41 of 424 dogs (9·8 per cent) bearing mammary tumours, although 186 (43·9 per cent) had histologically malignant tumours.

Carcinomas (87)

Subdivision of these epithelial tumours was made according to the predominant histological picture. It was possible to distinguish six types, as summarized in Table 1.

Squamous-cell carcinomas (5 cases). These were keratinizing squamous-cell carcinomas, possibly arising from the lining of a teat canal or of a major duct, although the occasional occurrence of squamous metaplasia in the glandular epithelium of benign tumours indicates another possible origin.

Duct carcinomas (19 cases). These were tumours derived from, and chiefly growing in, the duct system. The neoplastic changes, when their origin was detectable, could be seen to have occurred in normal ducts, or in the duct-like structures of benign tumours. The typical change, in an epithelium varying from low to tall columnar, was the formation of a number of folds in the lining of the ducts. These folds were usually without obvious stromal cores, or had only slight

supporting connective tissue, but in three tumours the stromal cores were more obvious and secondary folding was seen in the epithelium of the surface of the papillae. Small acini appeared in the folded epithelium, or more diffuse solid-looking areas of epithelium were seen, or the epithelium growing in the ducts had a cribriform arrangement.

TABLE I

CLASSIFICATION OF 87 CARCINOMAS OF THE MAMMARY GLAND

Incidence and distribution of metastases

Type	*Total number*	*Number with metastases*	*Distribution of metastases*		
			Lymph nodes	*Lungs*	*Elsewhere*
Squamous-cell carcinoma	5	1	0	1	0
Duct carcinoma	19	4	2	3	1
Adenocarcinoma	14	5	2	3	2
Sclerosing carcinoma	9	0	0	0	0
Solid carcinoma	30	6	5	3	2
Anaplastic carcinoma	10	5	2	4	0
	87	21	11	14	5

In a few places, there was squamous metaplasia, especially in two tumours, one of which was very superficially placed in the mammary gland. Signs of inflammation were often present. In the centre of many of the neoplastic duct structures there was a plug of necrotic material. Mitoses were numerous and there was outward invasion of the connective tissue, which was often necrotic, of the walls of the ducts. Dermal lymphatic permeation was present in places.

These duct carcinomas varied in size, being roughly oval in shape. Their edge was often well-defined when they were single nodular structures, but they generally consisted of multiple, greyish-white, firm masses embedded in an ill-defined brownish flabby tissue. Ulceration of the overlying skin was occasionally present.

Adenocarcinomas (14 cases). These were carcinomas consisting of glandular epithelium, in which ductal origin and intraductal growth were not prominent, and in which small structures with lumens were present. They formed nodular masses of varying size, resembling duct carcinomas macroscopically.

Sclerosing carcinomas (9 cases). These tumours showed marked sclerosis of the supporting connective tissue. Histologically, there

were one or more ill-defined lobular structures, of duct carcinoma or adenocarcinoma appearance, usually with lumens, embedded in a variably cellular, hyaline or fibrous tissue which in one tumour showed early chondrification and calcification. The epithelial elements tended to be more numerous at the periphery of the tumour and the sclerosing supporting tissue more obvious towards the centre, which was often necrotic. The tumours were in six instances well-defined, but the other three infiltrated the skin. They showed a greyish-white, firm, centrally cystic or necrotic cut surface.

Solid lobular carcinomas (30 cases). These showed solid lumenless sheets of cells, supported by fibrous trabeculae from which fine strands ramified, dividing the sheets of cells into small groups which might thus have a slight alveolar arrangement. In some instances, small acinar lumens were present and sometimes the sheets of cells were formed of closely-folded cords of epithelial cells: such tumours clearly originated from duct carcinomas.

The tumour cells tended to be polygonal, rounded, or rather spindle-shaped, or even ribbon-like. Some isolated cells were markedly larger than average and had large single nuclei, or were multinucleated. In places, squamous metaplasia was present and in two tumours small groups of cells had the morphology of sebaceous-gland epithelium. The cytoplasm of the tumour cells varied in intensity of staining and in some cases was quite clear. In 5 tumours, small vacuoles, or small droplets of mucin, were present in the cytoplasm. Some of the sheet-like masses showed central necrosis.

In at least three instances, the solid carcinoma lobules arose in benign tumours, while in a further 11 the tumours were in part duct carcinomas. A variety of types of cell may have been responsible for the solid lobular appearance. In some of the instances where duct-like structures were also present in the tumour, the tumour cells appeared to arise in the outer, rather than the lumenal, layer of epithelial cells. In two tumours of this kind subsequently examined, where the cells were of "clear" cytoplasm type, alkaline phosphatase activity was demonstrated in the cells, and the possibility exists that some of these solid lobular carcinomas are in fact of myo-epithelial origin.

These tumours formed single or multiple greyish-white masses, usually well-defined, of moderately firm tissue, although occasionally the consistency was like that of the canine seminoma. In a few instances, the overlying skin was ulcerated.

Anaplastic carcinomas (10 cases). The tumour cells formed, at most, very small ill-defined acinar structures. Both locally and in metastases, these tumours were accompanied by considerable fibrosis. They tended to form shield-like thickenings of a large part of one or both mammary chains, in the thoracic or abdominal region, with the formation of firm fibrous slabs of tissue in which tumour cells were often scarce. The tumours appeared to arise

rather diffusely in the mammary gland. Five of the 10 affected bitches were Scottish Terriers.

Sarcomas (73)

The 73 sarcomas could be divided into three groups viz. those that were sarcomas in which bony, osteoid, cartilaginous or chondroid tissue was formed (23 tumours), those which appeared to be of mammary gland origin, but were not osteosarcomas or chondrosarcomas (40 cases), and, thirdly, those sarcomas which, although they developed in a mammary gland (10 cases), were not of specific mammary origin. The division between the two first types is an arbitrary one, as the matrix-free areas of some osteosarcomas resembled that of other mammary sarcomas, but it was striking and easily made.

Osteo(chondro)sarcomas (23 cases). Histologically, the tumours showed plump, spindle-shaped, ribbon-like or tadpole-shaped cells, often with multinucleated giant cells, in a variable amount of bony or cartilaginous matrix (Figs. 12, 13 and 14), which was variably calcified. There was a tendency for an arrangement of the cells and matrix in a radiating fashion towards the periphery of the tumour. Bone, or osteoid, was present in at least 20 of the tumours with, in 10 cases, an admixture of cartilage, while the other three tumours appeared to be purely chondrosarcomas. The origin of these tumours was not clear, but some of the cells resembled those of the next type of sarcoma. The tumours were characteristically large, and oval in shape and had well-defined but irregular borders. The surface skin was stretched, sometimes adherent and often ulcerated. The centre of the tumours was usually cystic, the surviving tumour tissue being moderately to very firm in consistency and white in colour. Obviously bony or cartilaginous areas could usually be seen or felt, but sometimes the presence of the matrix was only detected histologically. Five of these tumours metastasized.

Other sarcomas of mammary origin (40 cases). There were, in addition to the 23 osteosarcomas, 40 sarcomas of mammary gland origin. At least 18 of them contained an intercellular mucoid matrix. The tumour cells were long, or short and plump, spindle-shaped or more ribbon-like. The mucoid matrix was generally scanty and patchily distributed: where it was abundant, the cells closely resembled the interposed cells of benign tumours. In two more recent examples of this kind, the cells showed marked alkaline phosphatase activity. These tumours were of varying size (up to 16 cm. long) and usually oval in shape. Their edge was not always well-defined, but the surface skin was generally not ulcerated. The tumour tissue was noticeably white in colour, rather glistening and moderately flabby, and showed patchy necrosis, often of the central part of the tumour, and much haemorrhage. Small mucoid cysts could sometimes be seen. Six of these tumours metastasized.

Other sarcomas (10 cases). These tumours, composed of more or

less rounded cells, and in 6 instances appearing to be lymphosarcomas, showed no clear indication of a specific origin from glandular tissue. An origin from the "round cells" of the kind found in benign tumours is possible. Two of these tumours metastasized.

Complex Malignant Tumours (*27*)

The nature of these 27 complex malignant tumours was uncertain. It is possible that some of them were actually truly mixed malignant tumours (carcino-sarcomas), that is, that they comprised malignant epithelium and malignant connective tissue or malignant myoepithelium. Other explanations for their histological structure, however, exist, viz. (i) the ostensibly mixed malignant tumour was merely an unusually cellular benign complex tumour; (ii) it was a carcinoma accompanied by active stromal growth of non-neoplastic tissue; (iii) it was a sarcoma, the distortion of included non-malignant epithelial structures simulating a carcinoma. It seems possible that these are complex tumours in which two components—epithelial and interposed cells—have undergone malignant change. In any event, their histology is sufficiently different from that of the carcinomas or sarcomas to warrant their being placed in a separate group.

The tumours showed histologically an intimate intermingling of epithelial and non-epithelial structures in varying proportions, both tissues showing features of malignancy. An epithelial component of glandular carcinoma type was always present, but there were often areas of squamous-cell carcinoma, particularly well-developed in 9 instances. The intermingling malignant non-epithelial components were of spindle-cell, fibro- or, in 5 cases, osteo-sarcoma type. The different tissues present are indicated in Table 2, and some of the appearances are illustrated (Figs. 15 and 16). Seven of the tumours metastasized.

TABLE 2

HISTOLOGICAL APPEARANCES IN 27 MALIGNANT COMPLEX TUMOURS

Table shows the histological appearances in malignant tumours not definitely classifiable as carcinomas or sarcomas.

Tumour No.	*Epithelial component*	*Non-epithelial component*	*Comment*
1.	Adenocarcinoma, chiefly ductal. Some squamous-cell areas	Cellular ossifying and chondrifying tissue	Cellular and apparently rapidly growing tumour, with epithelial malignancy; sarcoma not certain
2.	Adenocarcinoma	Spindle-cell, partly mucoid (?myoepithelial) tissue	Some of spindle cells may be carcinomatous. Active complex, with carcinoma and possible sarcoma
3.	Adenocarcinoma and some solid lobular carcinoma	Leiomyosarcoma	Probably malignant mixed
4.	Adenocarcinoma and squamous-cell carcinoma	Osteosarcoma	Ossifying tissue looks malignant and not just like rapid ossification

5.	Adenocarcinoma	Mucoid (?myoepithelial) tissue	Perhaps carcinomatous change in actively-growing complex tumour
6.	Squamous-cell and glandular carcinoma	Some ambiguous tissue	Not clearly sarcoma, but only partly carcinoma
7.	Squamous-cell and adenocarcinoma	Spindle-cell sarcoma (predominates in skin infiltration)	Possible carcino-sarcoma (lung metastases look like complex tumours)
8.	Adenocarcinoma	Sarcoma	An actively-growing complex tumour, and appears to be a carcino-sarcoma
9.	? Carcinoma	? Sarcoma	Could be active complex, but identification of cells uncertain
10.	Squamous-cell (and partly ductal) carcinoma	Osteosarcoma	Mixed malignant (both tissues present in a metastatic deposit in a lymph node)
11.	Adenocarcinoma	? active stromal reaction	Uncertain type
12.	Squamous-cell and spindle-cell carcinoma	? Possible spindle-cell sarcoma	Nature of spindle-cell component not certain
13.	Adenocarcinoma	Spindle-cell sarcoma	Could be carcino-sarcoma
14.	Duct carcinoma with squamous change	Actively-growing fibrous tissue	Could be carcino-sarcoma
15.	Adenocarcinoma, partly squamous	Sarcoma	Could be carcino-sarcoma
16.	Adenocarcinoma	Sarcoma	Could be merely an actively growing stroma, but looks more like sarcoma
17.	Duct carcinoma	Mucoid spindle-cell sarcoma	Some signs of carcino-sarcoma
18.	Squamous-cell and solid lobular carcinoma	Some spindle cells	Not a simple carcinoma
19.	Duct carcinoma	Osteosarcoma	Active bone production, but some spindle-cell sarcoma appearance between bone matrix
20.	Duct carcinoma	Osteochondro-sarcoma?	Or, active non-neoplastic production of bone and cartilage
21.	Duct carcinoma	Spindle-cell sarcoma	Also, some cartilage and bone
22.	Duct carcinoma	Spindle-cell tissue	Spindle cells could be carcinoma
23.	Adenocarcinoma	Spindle-cell sarcoma	Possible malignant mixed
24.	Adenocarcinoma	Spindle-cell sarcoma	Sarcomatous appearance predominates
25.	Carcinoma	? Chondrifying myoepithelium	Carcinoma with chondrogenesis
26.	Carcinoma	? mucifying myoepithelium	Mucifying solid carcinoma of unusual kind
27	Carcinoma Adenocarcinoma tending to solid type	? mucifying myoepithelium	Mucifying carcinoma?

Three of the animals with complex malignant tumours came to necropsy.

1. An 11-year-old mongrel had for some months had a diffuse neoplasm in the right anterior mammary gland with enlargement of the axillary

lymph node. After destruction neoplastic tissue was found in the right anterior mamma, the right axillary, right cubital and the suprasternal lymph nodes, and in the lungs, but not in the marrow of the long bones. The primary tumour and the lymph node deposits had the structure of squamous-cell carcinoma and adenocarcinoma along with spindle-cell tissue: some of the latter appeared to be anaplastic epithelium, while some was sarcomatous. In areas, chondrosarcomatous tissue was present. In the lungs, the tumour appeared partly as a papillary and acinar adenocarcinoma and partly as a sarcoma of spindle-shaped cells, with some myxomatous areas.

2. A tumour was removed from the third left mamma of a 14-year-old mongrel. The ulcerated and infected tumour (5 × 4 × 2½cm.) was white on the cut surface and almost bony in consistency, with patchy central haemorrhage. The bitch was destroyed 10 days after operation and showed local growth at the site of operation, many secondaries in the lungs, a few metastases in the diaphragm and one in the cortex of each kidney. The primary tumour was predominantly a sarcoma of spindle-shaped cells with mucoid matrix, but solid carcinoma areas were also present. The metastases in lung and kidney were spindle-cell sarcomas.

3. The left prescapular lymph node of a 10-year-old terrier-cross dog became enlarged, two years after the removal of an unexamined mammary tumour. The node (6 × 4½ cm.) was centrally cystic and of bony or cartilaginous nature at the edge. The lesion was partly a duct carcinoma, partly a spindle-cell sarcoma with bone and cartilage formation. Eight months later the bitch was destroyed, and showed the presence of malignant neoplastic tissue in the anterior suprasternal node, lungs, pericardium, pleura and left kidney. The lung and kidney lesions had the structure of adenocarcinomas, but the suprasternal node resembled the original prescapular node in structure.

Post mortem specimens were examined from three bitches. A mammary neoplasm was removed surgically from the left posterior gland of an 8-year-old Retriever. The tumour recurred locally and metastasized to the left axillary node. The nodal tumour had the structure of an adenocarcinoma, while the local tumour showed in addition some spindle-cell sarcomatous tissue.

A neoplasm was removed operatively from a nine-year-old Cairn. No recurrence was reported nor were metastases found after destruction. In a section of the tumour, adenocarcinoma and spindle-cell sarcoma tissue was present.

Portions of mammary tumour and a regional lymph node deposit were taken from a middle-aged bitch post mortem. The primary tumour had the structure of an osteosarcoma, but the lymph node deposit showed, alongside the osteosarcomatous growth, less extensive growth of a squamous-cell carcinoma.

Regional lymph nodes were available from four operation cases: nodal metastases were present in two.

A neoplasm had been present for a month in the right anterior mamma of the first, an 8-year-old terrier. The tumour had grown rapidly, and there was enlargement of the axillary node. The primary tumour, 5 × 4 cm., showed a small ulcer on the skin; the cut surface showed peripherally a whitish-yellow, irregularly lobulated tissue, with patchy glistening chondroid areas. The node (3½ × 2 cm.) had a similar appear-

ance, and was partly cystic. Histologically, the primary tumour showed adenocarcinomatous tissue, accompanied by ossifying fibrous tissue, which was in direct contact with the epithelium. In the node, both adenocarcinoma and chondrosarcoma were present.

From the second bitch, a 7-year-old mongrel, a mammary tumour and its regional node were removed; the tumour had been noted for one year, but had shown very rapid growth in one week before operation. The tumour (4 × 2 cm.) and the node (3 × 2 cm.) both consisted of white, firm, homogeneous tissue. The local tumour showed adenocarcinomatous tissue, along with partly mucoid spindle-cell sarcomatous tissue. The node tumour showed only adenocarcinoma tissue.

DISCUSSION

Mammary growths in the bitch have a great variety of histological structures and their full interpretation is very difficult. Having initially decided which of the cells present are of epithelial, myoepithelial or mesenchymal type, it is then necessary to decide which of these structures are abnormal and in what way. Since the mammary glands at different periods of the bitch's life undergo proliferative, functional and involutional changes, this is not easy. About the finer details of the causes and inter-relationships of the changes in these structures we still know too little, for in descriptions of the growth and involution of the gland, attention has been concentrated on the lumenal epithelium.

Even when it is concluded that the epithelium, the myoepithelium or the supporting connective tissue show abnormal proliferation, it must then be decided whether this is of a neoplastic nature. It is probable that some of the less complex benign lesions in the present series would be classed as hyperplastic or dysplastic by other workers (Romanelli, 1955). However, there is a gradation in complexity from simpler lesions to fully-developed benign, or even malignant, tumours, and when more or less well-defined nodular growths are present their classification as neoplasms seems justified on clinical grounds.

No agreement has yet been reached on the histogenesis of the pleomorphic complex tumours, in which there is, along with proliferated cells of frankly epithelial or frankly connective tissue type, a variable and sometimes predominant cellular component, closely related to the epithelium, which tends to become embedded in a matrix of mucoid or chondroid appearance.

Leaving on one side the theory that the "mixed" tumours, which are included in the group of benign complex tumours described here, are derived from embryonic rests, the mucoid and chondroid tissues have been considered to develop in relation to, and to be partly if not entirely produced by, cells which are of epithelial, myoepithelial or connective tissue origin.

Connective tissue origin. The simplest explanation of the presence of chondroid, as of mucoid, tissue is that it arises by metaplasia of fibrous connective tissue, which may be either a

constituent of the true (non-neoplastic) stroma, or the fibrous (neoplastic) component of a fibroadenoma. Those who have advanced this view of metaplastic mesenchymal origin include Jaeger (1910), Ball and Douville (1931), Auler and Wernicke (1932), Schmidt (1933), Möller (1941), Bloom (1954) and Moulton (1954). Even those authors who have attributed the bulk of the cartilage-like tissue in mixed tumours to a different origin have accepted, as does the writer, that some cartilage and bone of metaplastic origin may occur in the fibrous tissue of the tumour. The neoplastic epithelium has been considered to induce this stromal metaplasia, which would be in accord with the observations of Huggins and Moulder (1944) on the experimental induction of bone by mammary epithelium.

Epithelial origin. In contrast is the theory that mucoid and chondroid tissue arise in direct relation to the neoplastic glandular epithelium. Proponents of this view include Allen (1940), Mulligan (1949), Willis (1953) and Moulton (1954) (who, however, considers this to be a rare mode of origin). Although Allen considered the chondroid-like material present to be true cartilage, this is clearly a notion not easy to accept, as it would indicate an ectodermal origin of a tissue which is usually considered to be of mesodermal type. Those who find this a stumbling-block have considered that the properties of the chondroid material present would allow it to be called "pseudo-cartilage". Thus Willis considered that in the bitch mammary tumour, the microscopical appearances of cartilage might be simulated by epithelial cells scattered in mucinous secretion. It is difficult to avoid the conclusion that some of the material present in the complex mammary tumours of the bitch is true cartilage: for example, it is capable of undergoing endochondral ossification. The theory of epithelial origin is sometimes modified by the suggestion that the mucoid material produced by the epithelium is incorporated in, and changed by, the connective tissue to form cartilage.

Myoepithelial origin. The third cell which has been thought to be implicated in the production of mucoid, and chondroid, tissue in the mixed tumours is the myoepithelial cell. Richardson (1949) and Linzell (1952) have given accounts of myoepithelium.

The part played by the myoepithelium in the canine benign complex mammary tumour, first described by Peyron (1924) and Peyron, Corsy and Surmont (1926), has more recently been stressed by Hamperl (1939/40) and Biggs (1947). Biggs, for example, considered that in parts of the more complex tumours, reticulin, collagen, cartilage and bone may be deposited in what, from its general structure, appears to be a myoepithelial stroma. Hamperl similarly considered that the myoepithelium could be "umwandelt" into a tissue completely resembling cartilage or bone. Hamperl thought that the true glandular epithelium might also be involved in the formation of such tissue, not only indirectly by first becoming

transformed into myoepithelium, but also directly, so by-passing this stage of conversion into myoepithelium. It seems that Peyron *et al.* (1926) implicated both myoepithelial and connective tissue cells in the formation of cartilage and bone.

There are two other workers (Nieberle, 1933; Braemer, 1951) whose conclusions deserve consideration, as it seems that what they describe as epithelium in the one instance, or as connective tissue in the other, corresponds to the interposed cells of this present paper, so that, although ostensibly these authors fall into the "epithelial origin" or the "connective tissue origin" camps, their observations are compatible with a myoepithelial origin of the mucoid and chondroid tissue. Nieberle (1933) described a "reticulated epithelial tissue", which became embedded in a mucoid matrix to give the appearance of cartilage and an accompanying additional myxomatous change in the connective tissue stroma. He described the reticulated epithelium as lying inside the basement membrane, at any rate in early cases, and, although it cannot be concluded that cells lying in this position must be myoepithelial, this seems their most likely nature. Braemer (1951) described a cellular tissue of the same kind, but considered it to be, not epithelium, but a young connective tissue arising intra-alveolarly: this again could well be myoepithelial. It seems possible that Nieberle has emphasized the epithelial aspect of the cells involved, while Braemer has been impressed by their apparent production of tissue of mesodermal type.

From the present study of benign complex mammary tumours, the opinion was formed that there is in them often a predominant proliferation of cells, with characters indicating that they are myoepithelial cells often tending to become embedded in a mucoid and chondroid matrix which they produce at least in part. Identification of the cells in question as myoepithelium has been supported by histochemical demonstration of alkaline phosphatase activity in them. Catellani (1956) has described the alkaline phosphatase activity of canine mammary tumours and Erichsen (1955) other histochemical aspects.

A difficult problem is that of the significance of the mucoid and chondroid changes. It is possible that the presence of the mucin is not just a fortuitous degenerative change, but is related to normal growth and involutional changes in the gland. Perhaps the mucin accumulates not because it is being formed in excess but because it is not being destroyed at a normal rate. Elliott and Turner (1950) have suggested that a mucolytic enzyme or collagenase is elaborated by cells of the growing mammary gland of the rat and they relate this to the invasion of connective tissue by the growing glandular buds. It would be worth while trying to see whether the epithelium or myoepithelium normally produce mucinase.

As regards the nomenclature of the benign complex tumours, the phrase "mixed tumour" may, as suggested by Sheldon (1943), be usefully applied to tumours composed of epithelial and myoepi-

thelial cells and not restricted to its ordinary sense of a fibroadenoma. Mulligan (1949) stated that the "term mixed tumour is appropriate, not only because the neoplasm is derived from both ectodermal and mesodermal elements, but also because of the heterogeneous character of the epithelial, myoepithelial and connective tissue proliferations observed". The writer has preferred to use the term "complex tumour", and in reporting the histology to the veterinary surgeon concerned, has used some such description as "complex tumour, with predominant myoepithelial component, and mucoid (or chondroid, or bony) transformation". As there is an indication that these benign complex tumours may show malignant change, particularly in the myoepithelial component, special attention is given to that tissue in assessing prognosis. Since there seems little advantage in sharply separating off the papillary cystadenomas as they may show varying amount of "mixed tumour" tissue, lesions of both kinds are included in the term "complex tumour".

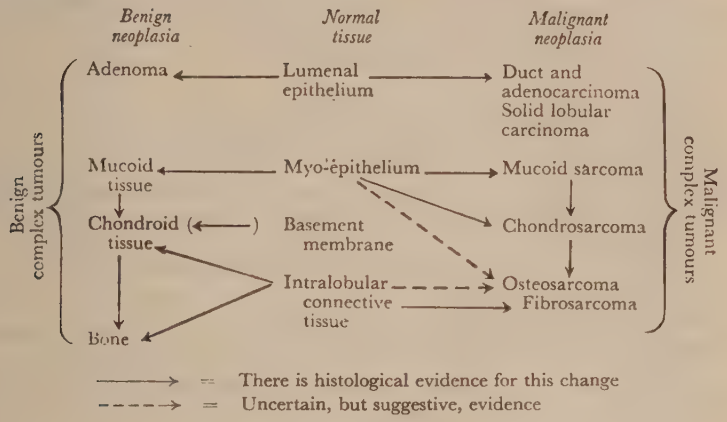
In the series of malignant tumours, there was a high incidence of tumours of non-epithelial or of complex structure. It seems possible that some of the sarcomas were malignant myoepithelial tumours with a "mesenchymal" aspect and that some of the solid lobular carcinomas were malignant myoepithelial tumours with an "epithelial" aspect. If it is accepted as possible that the myoepithelium, although of ectodermal (? neuroectodermal) origin, has the power of forming tissues of mesenchymal type there is no intrinsic difficulty in regarding the fibrosarcomas, mucoid sarcomas, chondrosarcomas and osteosarcomas as being possible malignant derivatives of the myoepithelium. There is an analogous problem of the Schwann cell and its neoplastic derivatives (Evans, 1956).

In this series, only about 10 per cent of the tumours proved themselves to be malignant by metastasizing—the same percentage as that estimated by Dobberstein and Matthias (1942). Further studies are needed before a satisfactory grading of malignant tumours for prognostic purposes can be achieved, but it is clear that a malignant histology does not entail a necessarily hopeless prognosis.

It may be useful to sum up in diagrammatic form the probable and possible inter-relationships of the benign and malignant tumours of the bitch's mammary gland and of the normal structures from which they originate. The diagram is an over-simplification of the situation. It leaves out of account, for example, the interposition of a hyperplastic stage between normality and neoplasia, and also the possible inter-relationships of the benign and malignant tumours.

The aetiology of these common neoplasms of the bitch's mammary gland is still not clear (Bloom, 1954). Since the myoepithelium appears to play a significant role in a large proportion of mammary neoplasms of the bitch, a study of the stimulus, presumably

hormonal, which causes normal growth of this type of cell is indicated.



CONCLUSIONS

An account is given of the pathology of mammary tumours from 424 bitches. There were 249 benign tumours. Of these, 11 were of connective tissue type and 8 were simple adenomas. The other 230 benign tumours showed varying degrees of complexity, generally with proliferation of "interposed cells", that is, of cells characteristically interposed between lumenal epithelium and basement membrane or intralobular connective tissue. There was often a formation of mucoid, chondroid, or bony tissue: 40 of the benign complex tumours contained bone, an additional 63 contained cartilage but no bone, and a further 67 showed mucoid tissue, but no cartilage or bone.

Reasons are given, including the histochemical demonstration of their alkaline phosphatase activity, for holding that the interposed cells are myoepithelial cells.

These complex tumours include papillary cystadenomas and mixed tumours, and their histogenesis is discussed. It is suggested that there is a predominant proliferation of myoepithelial cells, which tend to become embedded in a mucoid, or chondroid, matrix, which they produce at least in part. Some cartilage is formed in relation to myoepithelial cells, some by metaplasia of connective tissue. The bone in the tumours appears to be formed either by endochondral ossification of preformed cartilage, or by intramembranous ossification in the connective tissue of the tumour.

The 187 malignant tumours were classified as carcinomas (87), sarcomas (73) and complex malignant tumours (27). Metastases were present in 41 of the 424 bitches.

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LEGENDS TO ILLUSTRATIONS

All the tumours are of mammary gland origin.

Fig. 1. Benign complex tumour from 5-year-old Spaniel bitch, showing cartilage (at left of field) with no definite fibrous perichondrium or capsule. In the centre and right of the upper half of the field, multiple foci of proliferated interposed cells appear as rounded masses between the acinar structures. H. & E. × 43.

Fig. 2. Same tumour, showing the middle region of the edge of the cartilage in Fig. 1 under higher magnification. There is no perichondrium or fibrous capsule around the cartilage, which merges with no definite boundary into a cellular tissue, with a loose foamy (mucoid) matrix containing stellate cells (centre). H. & E. × 190.

Fig. 3. Cartilaginous benign complex tumour of a 13-year-old mongrel bitch, showing 3 epithelial structures (top centre, lower centre and lower left) embedded in cartilage, the matrix of which is not separated by a fibrous perichondrium or capsule from the epithelium. H. & E. × 280.

Fig. 4. Bony benign complex tumour from 3-year-old mongrel bitch, showing endochondral ossification, with a cartilage mass (upper part of field) in which there is a linear arrangement of cartilage cells, simulating an epiphyseal plate, and a mesh of bone embedded in cellular tissue (centre). H. & E. × 38.

Fig. 5. Bony benign complex tumour, from a 7-year-old cross-bred bitch, showing glandular structures (top), cartilage (left), and bone which is partly apposed to the surface of the cartilage, and (centre and below) tissue recalling myeloid and fatty bone marrow in appearance. H. & E. × 45.

Figs. 6–10 are prepared by the modified coupling azo dye method (Pearse, 1953) to show alkaline phosphatase activity. "Positive" cells appear red in the original preparations (which are counterstained with haematoxylin) and black in the reproductions. The contrast in the original is greater than in the prints shown here.

Fig. 6. Benign complex tumour, after ½-hour incubation, showing a group of alkaline phosphatase "negative" interposed cells, which are partly embedded in a matrix to resemble cartilage. These interposed cells occupy a position corresponding to that of myoepithelium in relation to lumenal epithelium. The section is taken near the bifurcation of a tubular epithelial structure, and parts of both branches are seen. The main lumen is seen in the right half of the field. On the right of this lumen can be seen (negative) lumenal epithelium and (positive) myoepithelium; on the left of this lumen can be seen lumenal epithelium, and immediately outside this, not (positive) myoepithelium, but a group of proliferated cells, classed as "interposed" (in this case negative). × 170.

Fig. 7. Benign complex tumour from middle-aged mongrel bitch, showing a sheet of cells (right half of section), some of which show alkaline phosphatase activity (2 hr. incubation). These cells are continuous with masses of cells showing alkaline phosphatase activity, which are themselves continuous with (positive) myoepithelial cells (upper and lower left part of the field). The lumenal epithelial cells (left) are negative. × 200.

Fig. 8. Benign complex tumour, with early cartilage formation. The section illustrates the ambiguity as regards position of the cells in the cartilage matrix in relation to the myoepithelium. In the centre of the field, the proliferated cells in the chondroid matrix appear to be clearly outside (to the left of) the alkaline phosphatase positive myoepithelium, but (lower centre) cells which merge into the matrix, and are on the other hand continuous with positive myoepithelial cells, are themselves partly positive. ½-hour incubation. × 170.

Fig. 9. Benign complex tumour (papillary cystadenoma) of 10-year-old mongrel bitch, with alkaline phosphatase positive cells (½-hour-incubation) shown in sweeping bundles in the cores of the papillae (lower right particularly). × 160.

Fig. 10. Mucoid benign complex tumour, showing groups of alkaline phosphatase positive cells (½-hour incubation) in mucoid masses, and positive myoepithelial cells in duct structure. The sites of alkaline phosphatase activity appear black—seen particularly in two spidery cells (just to the right of the centre). × 184.

Fig. 11. Cartilaginous benign complex tumour of five-year-old Labrador bitch, showing multiple white chondroid lobules aggregated to form an elongated mass below teat. Many of the lobules are closely similar in size (scale, left, in mm.).

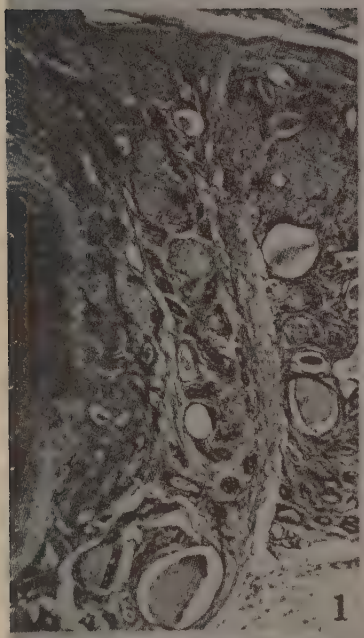
Fig. 12. Chondrosarcoma of a 13-year-old mongrel bitch. The tumour cells in part form sheets or cords (lower part of field). H. & E. × 200.

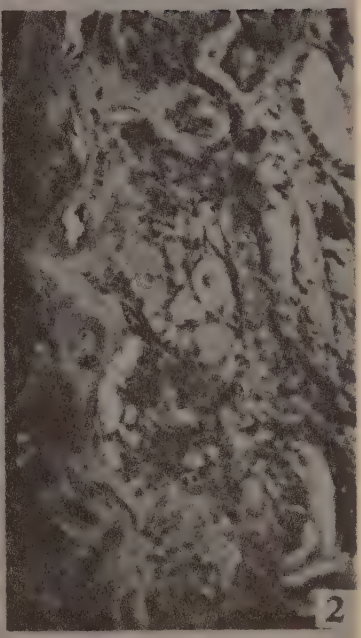
Fig. 13. Section of metastasizing mammary osteosarcoma of a 16-year-old mongrel bitch. H. & E. × 40.

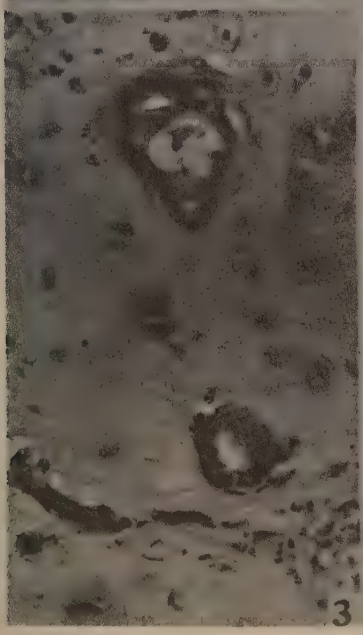
Fig. 14. Metastatic lesions of a mammary osteosarcoma in the lungs of an 11-year-old mongrel bitch. H. & E. × 60.

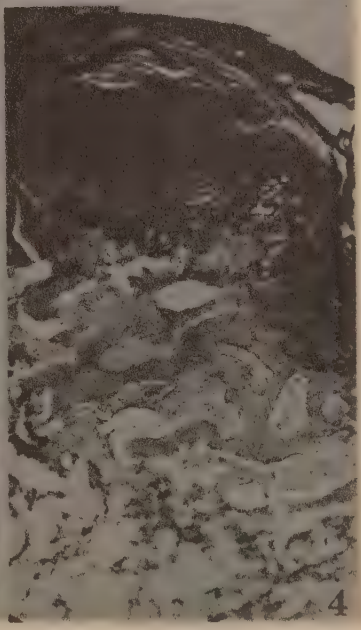
Figs. 15 and 16. Complex malignant tumour of an 8-year-old mongrel bitch, showing the primary lesion (fig. 15) and a lymph node deposit (fig. 16). In the primary lesion, the appearances are of a duct carcinoma (above) and a possible osteosarcoma (below), and in the lymph node of a duct carcinoma (left and above) and a possible chondrosarcoma (right). H. & E. × 60 for each section.

Plate I









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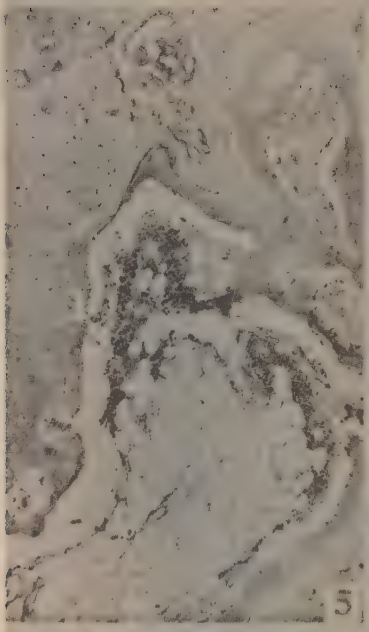

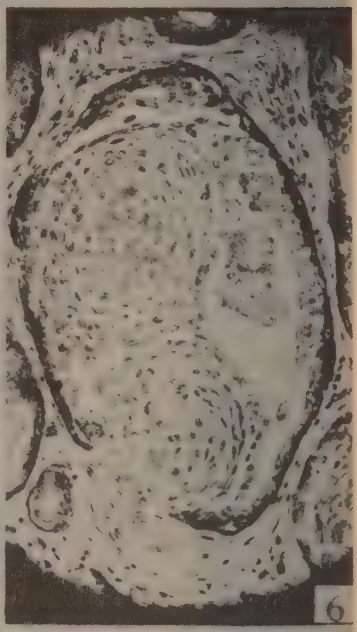

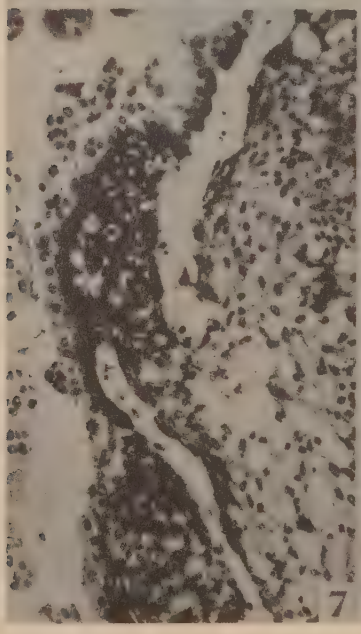

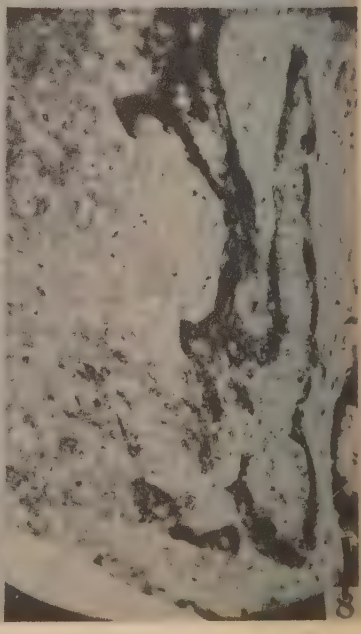

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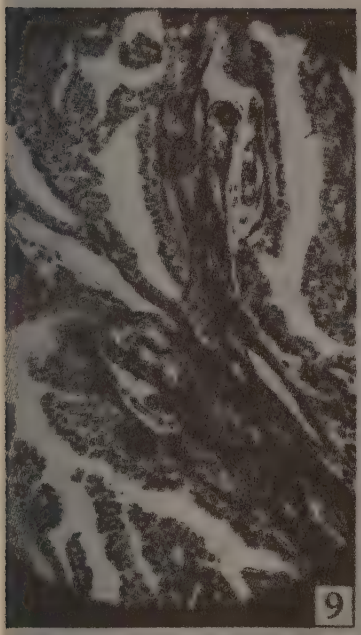

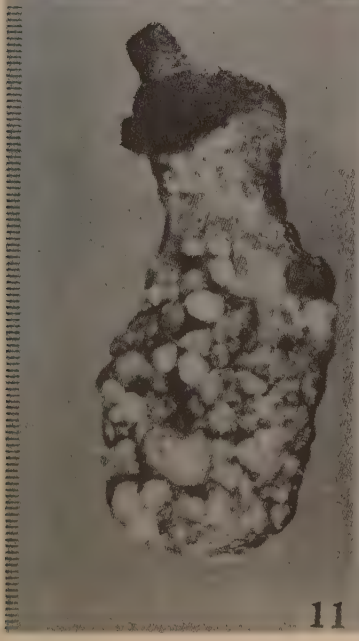

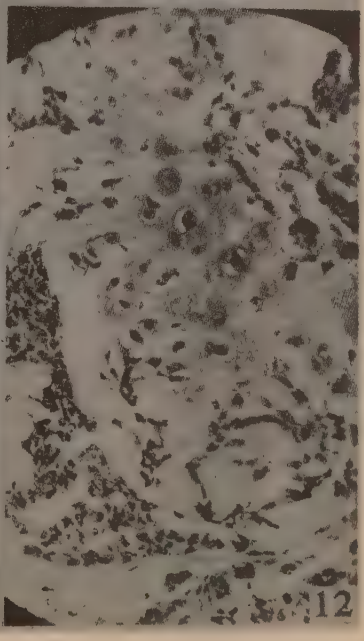

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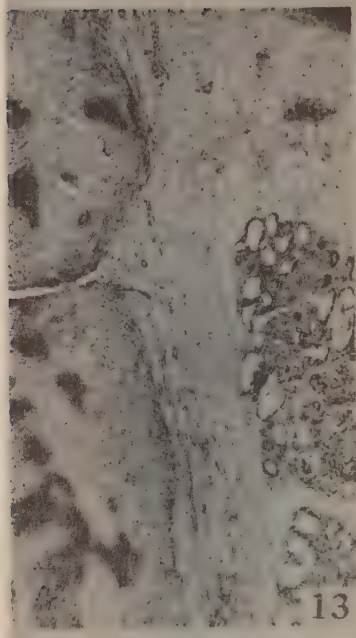

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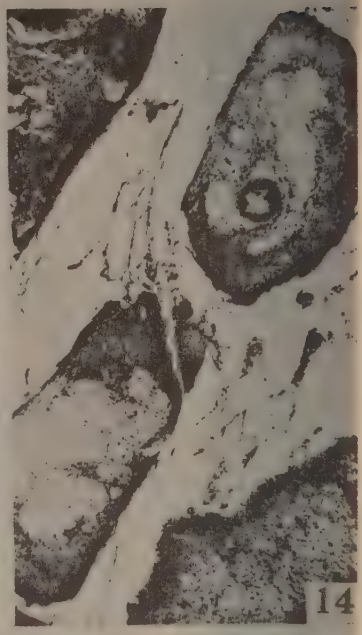

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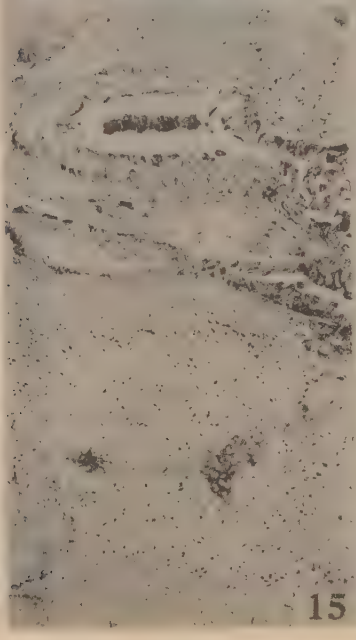

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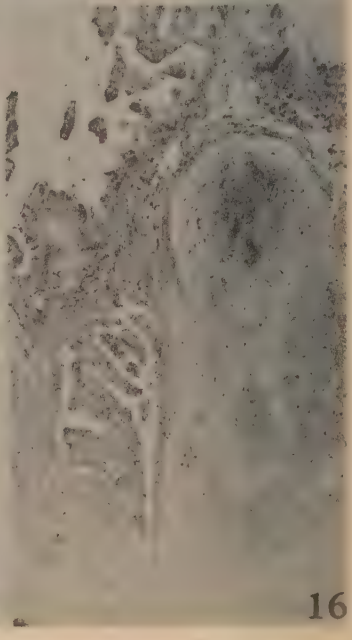

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CONTAGIOUS PUSTULAR DERMATITIS

IV. Immunological Reactions

By

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INTRODUCTION

In contrast to the considerable attention paid to immunity and cross-immunity tests in sheep the in vitro reactions between the virus and antiserum have attracted little attention. Much of this neglect was perhaps attributable to the difficulty of preparing suitable antigens, for previous to the present inquiry, crude scabs or excised lesions were the usual sources of the antigen. In the present investigation, it was shown that using concentrated and relatively pure suspensions of the virus, specific agglutination, complement fixation and in vitro inactivation with antisera can be demonstrated. With a soluble antigen derived from the virus suspension and infected tissues, it was also possible to demonstrate a flocculation reaction.

METHODS

Elementary body (e.b.) suspensions. The method of preparation has been described. Stock suspensions which had been stored at 0 — 4°C. for several weeks were used after re-washing in buffer solution (Abdussalam and Cosslett, 1957).

Elementary body supernatant fluid was the supernatant fluid obtained by the angle-centrifugation of e.b. suspensions stored for varying periods. The fluid was slightly hazy but free from e.bs. as shown by microscopic and infectivity tests.

First supernatant. This is analogous to the 'first supernatant' of vaccinia (Parker and Rivers, 1935). It was obtained from the first spinning in the angle-centrifuge during the course of preparation of e.b. suspensions from scrapings of infected sheep skin. It was slightly hazy when first prepared, but a coarse precipitate appeared on storage in the refrigerator. It was, therefore, clarified by prolonged spinning in the angle centrifuge before use.

Immune sheep serum. Two lambs which had recovered from an experimental skin infection were inoculated intravenously each with 0.25 c.c. e.b. suspension (opacity, Brown No. 4) on three successive days. After an interval of 10 days, the injections were repeated on 3 successive days and the animals bled 10 days afterwards. Their sera were filtered through a Seitz E.K. pad, stored at low temperature and used separately or mixed.

Immune rabbit serum. Two adult rabbits convalescing from C.P.D. were injected in the same manner as the foregoing lambs with 0·2 c.c. of the e.b. suspension on each occasion and bled a week after the last

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injection. Two more convalescent rabbits were each given four intravenous injections of 0·5 c.c. e.b. suspension at 3-day intervals and bled 3 days after the last injection. Their sera were pooled, one half being Seitz-filtered and the other half preserved with merthiolate (1 in 10,000). No difference was noticed in the potency of the sera prepared by these two methods.

Convalescent sera were obtained from sheep 3 to 4 weeks after infection and from rabbits two weeks after infection. As a rule, sera from 3 or 4 animals were pooled and used fresh or after storage following filtration or treatment with merthiolate. A human convalescent serum was obtained from a volunteer who had been infected twice before bleeding. He was bled 31 days after the first and 7 days after the second infection.

Recovered sera were obtained from animals at varying intervals after infection.

Normal sera were obtained from animals which so far as is known, had no history of C.P.D. or other skin disease. Normal human sera were kindly supplied by Dr. R. R. A. Coombs but the history of persons from whom they were obtained was not available.

Haemagglutination

Methods

The virus preparations consisted of e.b. suspensions or first supernatant in C.P.D. and saline extracts of 3-day chorio-allantoic lesions in the case of vaccinia. The latter were prepared by a method analogous to that used by Burnet and Boake (1946). The haemagglutination tests in general followed the technique of Nagler (1942). Dilutions were made in saline using a standard volume of 0.25 c.c., one volume of one per cent red-cells being subsequently added. The pattern of depositing cells at the bottom of tubes was examined with a mirror every 15 minutes for up to one hour, the test being carried out at room temperature. Some tests with fowl red-cells were also carried out by allowing the virus-cell mixtures to interact for 30 minutes at 37°C. before shaking and setting at room temperature (Burnet and Boake, 1946), but this did not alter the result. All cell suspensions used were prepared after washing in three changes of saline and used within 24 hours of collection.

In haemagglutinin inhibition tests with sera, a standard volume (0.25 c.c.) of serum dilution was incubated at 37°C. for ½ hour with an equal volume of the membrane extract dilution. One volume of cell suspension was then added and the result read at intervals during one hour.

Results

The e.b. suspensions as well as first supernatant of C.P.D. in dilutions of ½ to $\frac{1}{64}$ failed to agglutinate the following species of red-cells (the numbers in brackets indicate the numbers of individuals of the species employed): fowl (9), pigeon (3), sheep (4), goat (1), horse (1), calf (1), rabbit (3), guinea-pig (2) and mouse (2). The pattern of depositing sheep cells at first gave an impression of forming a sheet at the bottom of the tube but the usual ring and central spot of the 'negative' pattern soon appeared, both in the test and control tubes. This was ascribed to the small size of sheep

cells. The fowl cells used included four vaccinia-positive samples. Immune C.P.D. sera from sheep and rabbits failed to inhibit haemagglutination of fowl red cells by vaccinia virus.

Experiments with sensitised human and bovine red-cells. Pickles (1946) showed that Rh positive human cells sensitised with "incomplete" Rh antibodies were agglutinated when cholera filtrate was added. Chu and Coombs (1947) observed that Rh positive cells, treated with influenza and related viruses, became susceptible to agglutination with the foregoing antibody. In the present investigation, 4 per cent O R_1 R_1 cells sensitised with the 'incomplete' anti-D Rh antibody were treated with varying dilutions of C.P.D. e.b. suspension and C.P.D. first supernatant. Unsensitised cells were similarly treated as controls. Naked-eye and microscopic examination at the end of 3 hours' contact failed to show any evidence of agglutination. Two per cent suspensions of ox red-cells sensitised with the Paul-Bunnel antibody, as well as unsensitised cells, likewise remained unaffected by the two C.P.D. virus preparations mentioned above.

Agglutination

Ledingham (1931, 1932) used a hanging-drop micro-agglutination technique for the agglutination of vaccinia particles. This test has not been employed, however, by previous workers on C.P.D. as they did not have e.b. suspensions for this purpose. In the present investigation, a capillary micro-agglutination method was found to be equally economical and more satisfactory, as it was possible to demonstrate specific agglutinins to a high titre in immune and convalescent sera.

Methods

Fresh e.b. suspensions were centrifuged to remove any aggregates that may have formed during storage. The supernatant containing the dispersed e.bs. was then washed in two changes of buffer solution and re-suspended in sufficient McIlvaine's buffer solution to match Brown's opacity tube 4 or 5. Suspensions washed more than two days previously were not used without further washing.

A capillary, fitted with a small teat and open at both ends, was marked with a glass pencil at about one-third its length from one end. The serum dilution was sucked up to the mark and discharged into the cavity of a china block such as is commonly used with a capillator. A similar amount of the e.b. suspension was mixed thoroughly with the serum and was then sucked up into the same capillary tube which was sealed at one end in a small flame and stuck upright in a block of plasticine. The tubes were kept at room temperature and the result was read 18 hours later. Agglutination was shown by a brownish-white flocculate at the bottom of the column of fluid in the capillary, the supernatant being clear. In capillaries which were not quite vertical the floccules were seen on one side of the tube, but slowly settled to the bottom if the tube was placed in a vertical position.

Results

The results of several tests are summarised in Table 1. The titre

of the serum in the second column is the highest dilution in which complete agglutination leaving a clear supernatant was observed, the dilution referring to the serum part only and not to the total mixture in the tube. Normal human, cattle and rabbit sera did not show any agglutinins while normal sheep sera showed them to a low titre. Horse serum, however, had a natural agglutinin for the virus. Convalescent and particularly the immune sera in all susceptible species showed a rise in the titre which in man was detectable in one case 132 days after natural infection. Vaccinia convalescent rabbit serum showed only a low titre. Sheep in the early stages of the disease or 5 months after recovery showed no rise in the titre.

TABLE 1

AGGLUTINATION TITRES OF C.P.D. AND OTHER SERA

Serum	*Titre*
Sheep sera	
Healthy (2)	1/8 1/16
Infected 5 days previously	1/4
C.P.D. convalescent	1/64
C.P.D. recovered 5 months previously	1/16
C.P.D. immune (2)	1/512 1/1,024
Rabbit sera	
Healthy (2)	nil nil
C.P.D. convalescent	1/64
C.P.D. immune (2)	1/256 1/512
Vaccinia convalescent	1/2
Human sera	
Healthy (3)	nil nil nil
C.P.D. convalescent from re-infection	1/128
C.P.D. recovered, 132 days previously	1/64
Other sera	
Normal horse serum (2)	1/256 1/256
Normal cattle serum	nil

Precipitin

This test has been usefully employed in the study of variola and vaccinia (Gordon, 1925; Craigie and Tulloch, 1931), but Glover (1932-33) and Manley (1934) both failed to demonstrate flocculation in C.P.D. with Gordon's technique. In the present studies, similar results were at first obtained when extracts of scabs and convalescent sera were used as in vaccinia (Tulloch, 1940). Subsequently, however, specific flocculation was observed when immune sera and first supernatant or buffer solution in which e.bs. had been suspended was used. Because of the weak reaction shown by convalescent sera and none at all by scab extracts, this test was not considered to be of the same diagnostic value as in vaccinia.

Methods

E.b. suspensions which had been stored in the cold-room for 2 to 8 months were centrifuged. The slightly opalescent supernatant, free from e.bs. was used as an antigen. The "first supernatant" was also used, but was always centrifuged to remove particulate matter. The sera were used undiluted or at a 1 in 4 dilution in buffered saline prepared by diluting McIlvaine's buffer with 50 volumes of normal saline. The reagents (0·05 c.c.) after mixing were kept at room temperature and examined at intervals with oblique lighting against a black background. In positive cases, a white ring usually appeared at the junction of the two fluids in an hour, after which the precipitate became coarser and sank to the bottom. The tubes were then shaken and the final readings taken the next day.

Results

In the first test rabbit sera were tested with three undiluted antigens and the results are shown in Table 2. In a further test, two-fold serial dilutions of supernatant fluid obtained from a 2-month old e.b. suspension were tested with various sera (Table 3). Dilutions of the 'first supernatant' (1) were used in another test with sheep sera and the results are shown in Table 4. In a single test, in which a rabbit vaccinia 'first supernatant' was tested against vaccinia convalescent and C.P.D. immune rabbit sera, flocculation was observed only with the vaccinia serum.

TABLE 2

UNDILUTED ANTIGEN

Rabbit sera tested (1 in 4)	*Antigens*			*Saline control*
	E.b. fluid (8-month old)	*First supernatant (1)*	*First supernatant (2)*	
C.P.D. immune	+ + +	+ + +	+	—
C.P.D. convalescent	+ +	+	+	—
Vaccinia convalescent	—	—	—	—
Normal rabbit	—	—	—	—
Saline only	—	—	—	o

+ + + = flocculation easily seen with naked eye.
+ + = flocculation discernible without hand lens.
+ = flocculation discernible only with hand lens.
— = no flocculation. o = not tested.

Complement Fixation

This test has been employed in diagnosis and experimental study of several virus diseases (Craigie, 1939). Glover (1932-33) used the

method in C.P.D., but had to subject his antigen (scab extract) to septic maceration for seven days before he obtained consistent results. Manley (1934) failed to observe any specific fixation of complement. Rottgardt, Aramburu and Pirazzi, (1949) and Macdonald (1951) have reported specific complement fixation using extracts of dry scabs and dried skin scrapings respectively. In the present investigation, using more refined antigens, it was possible to demonstrate specific complement fixation.

TABLE 3
SERIAL DILUTIONS OF ANTIGEN

Sera tested (1 in 4)	*Dilutions of e.b. supernatant fluid*						*Saline control*
	2	*4*	*8*	*16*	*32*	*64*	
Rabbit sera							
1. C.P.D. immune	+ + +	+ + +	+ +	+	—	—	—
2. C.P.D. convalescent	+ +	+ +	—	—	—	—	—
3. Vaccinia convalescent	—	—	—	—	—	—	—
Sheep sera							
4. C.P.D. immune (1)	+ + +	+ + +	+ + +	+ +	+ +	—	—
5. C.P.D. immune (2)	+ + +	+ + +	+ + +	+	+ +	—	—
6. C.P.D. convalescent	+	—	—	—	—	—	—
7. Healthy sheep	—	—	—	—	—	—	—
8. Saline control	—	—	—	—	—	—	

Notes : (1) Dilutions of antigen refer to the quantity of antigen itself and not to the total mixture in the tube.
(2) The significance of signs is the same as in Table 2.

TABLE 4
FIRST SUPERNATANT

Sheep sera tested (1 in 4)	*Dilutions of 'first supernatant'*						*Saline control*
	2	*4*	*8*	*16*	*32*	*64*	
1. C.P.D. immune	+ + +	+ + +	+ + +	+	—	—	—
2. C.P.D. convalescent	+ +	+	—	—	—	—	—
3. Healthy sheep	—	—	—	—	—	—	—
4. Saline control	—	o	o	o	o	o	o

Note: The significance of signs is the same as in Table 3.
o = not done.

Methods

In general, the technique of Craigie and Wishart (1934) was adopted, but the dose of complement and conditions of incubation of mixtures of antigen, serum and complement had to be ascertained. The sera were inactivated at 56°C. for 30 minutes. No difficulty arose from natural haemolysins and no anticomplementary effect was observed in the dilutions of reagents ordinarily used.

The complement was the serum of two guinea-pigs preserved at low temperature. Craigie and Wishart (1934) used 3 m.h.d. of complement and incubated the test mixtures at 4°C. for 16 hours. As the increased sensitivity may have been largely due to a fall in the titre of the complement, it was decided to use 1·5 m.h.d. and to incubate the test mixtures at room temperature for 2 to 2½ hours before adding the haemolytic system. Incubation at 37°C. for ½, 1 and 2 hours with 3 m.h.d. of complement was found to be unsatisfactory. In the test, 0·2 c.c. of two-fold serial dilutions of antigen starting with 1/50; 1·5 m.h.d. of complement and a constant dilution of serum were kept at room temperature. Then, 0·25 c.c. of haemolytic system comprising 4 per cent sheep red cells sensitised with 4 m.h.d. of haemolysin was added and the tubes incubated at 37°C. for 30 minutes. In reading the test the degree of haemolysis is expressed by the figures 3, 2, 1 and 0. 'Three' signifies no haemolysis and '0' signifies complete haemolysis. The titre of the antigen is expressed as the highest dilution showing complete fixation of the complement.

Results

Antigen concentration. The effect of using serial dilutions was checked with four antigens (Table 5). A one per cent concentration of immune rabbit serum was selected at random.

TABLE 5

VARYING DILUTIONS OF ANTIGEN

Antigen	*Antigen dilutions*						*Controls*		
	50	*100*	*200*	*400*	*800*	*1600*	*Antigen*	*Serum*	*Complement*
(1) C.P.D. e.b. suspension	3	3	3	3	2	0	0	0	0
(2) Supernatant of (1)	3	2	1	0	0	0	0		
(3) C.P.D. first supernatant	3	3	3	3	3	2	0		
(4) Gradocol (0·7μ) filtrate of (3)	3	3	3	2	0	0	0		
(5) Vaccinia e.b. suspension	0	0	0	0	0	0	0		

Symbols have been explained in the text.

TABLE 6

VARYING DILUTIONS OF IMMUNE RABBIT SERUM

Serum dilutions	*Antigen dilutions*										*Controls*		
	50	*100*	*200*	*400*	*800*	*1600*	*3200*	*6400*	*12800*	*25600*	*Antigen*	*Serum*	*Complement*
1/10	3	3	3	3	3	3	3	3	3	3	0	2	0
1/25	3	3	3	3	3	3	3	2	1	0		0	
1/50	3	3	3	3	3	3	2	0	0	0		0	
1/100	3	3	3	3	2	1	0	0	0	0		0	
1/200	2	0	0	0	0	0	0	0	0	0		0	
1/400	0	0	0	0	0	0	0	0	0	0		0	

Serum concentration. The concentration of immune serum was then varied to find the optimal concentration (Table 6). The antigen used was C.P.D. first supernatant which had fixed complement in the maximum dilution in the foregoing test. It was necessary to determine the anticomplementary activity of the sera. The sample used in Table 7 was somewhat anticomplementary in a 1/10 dilution. The optimal dilution was therefore considered to be 1/25. Some other sera were not anticomplementary at a 1/10 dilution and were used at that concentration. When sera from rabbits, sheep and man were tested it was found that the titre of immune sera was the highest and convalescent sera varied. Fixation by human sera was weak and normal sera of all the three species as well as vaccinia serum showed little or no fixation (Table 7).

Effect of treating the antigen. The extent of fixation by various antigens has already been shown in Table 6. The effect of boiling and washing e.b. suspensions using immune rabbit serum (1/25) is set out in Table 8. It will be seen that washing and boiling had little effect and that supernatant fluid from a 2-month old suspension was as active as the orginal suspension. It appears therefore that the antigen (or antigens) responsible for this reaction is contained in the elementary bodies as well as in the fluid in which they have remained for some time. The power to fix complement is not destroyed by boiling.

Neutralisation

Aynaud (1923) was unable to demonstrate any inactivating effect of convalescent sera on the C.P.D. virus in crude scab suspensions. Glover (1932-33) pointed out that the virus in such crude suspensions may have been mechanically protected and did not therefore come in contact with the antibody in the serum. Accord-

ingly, he used an autolysate of the crusts as infective material and demonstrated the neutralising effect of an immune sheep serum and, to a lesser extent, of a sample of convalescent sheep serum. Later, Manley (1934) confirmed these findings. All these workers titrated their virus-serum mixtures on scarified sheep skin, a procedure not altogether ideal for such work. In the present investigation the question was re-examined with washed e.b. suspensions as infective material and the intradermal inoculation of rabbits as a method of titration, both of which were obviously better than the older procedures. Neutralising antibodies were demonstrated in immune as well as convalescent sera.

TABLE 7

COMPLEMENT FIXATION WITH VARIOUS SERA

Serum end dilution	*Antigen dilutions*									*Controls*		
	50	*100*	*200*	*400*	*800*	*1600*	*3200*	*6400*	*12800*	*Anti-gen*	*Serum*	*Comple-ment*
Rabbit sera												
C.P.D. immune 1/25	3	3	3	3	3	3	3	2	1	0	0	0
C.P.D. convales-cent 1/25	3	3	2	1	0	0	0	0	0		0	
Vaccinia con-valescent, 1/25	2	1	0	0	0	0	0	0	0		0	
Normal, 1/10	0	0	0	0	0	0	0	0	0		0	
Sheep sera												
C.P.D. immune, (1) 1/10	3	3	3	3	2	0	0	0	0		0	
C.P.D. immune, (2) 1/25	3	3	3	3	3	2	0	0	0		0	
C.P.D. convales-cent, 1/25	3	3	2	0	0	0	0	0	0		0	
Normal, 1/25	2	0	0	0	0	0	0	0	0		0	
Human sera												
C.P.D. convales-cent, 1/25	3	3	2	0	0	0	0	0	0		0	
C.P.D. convales-cent, 1/10	3	2	2	0	0	0	0	0	0		0	
C.P.D. re-covered, 1/10	2	0	0	0	0	0	0	0	0		0	
Normal, 1/10	0	0	1	1	0	0	0	0	0		0	

Methods

Serial 10-fold dilutions of the stock or diluted washed e.b. suspension (U.F. strain) were prepared in buffer solution and an equal amount of a

1 in 3 saline dilution of the serum was added to each tube. In view of the futility of incubating serum-virus mixtures in vaccinia and some other virus diseases (Andrewes, 1928) this procedure was dispensed with in the present experiments. Of each dilution 0·2 c.c. was inoculated intradermally on the skin of a rabbit. Usually one dilution was inoculated at one spot, but sometimes duplicate injections of a series were made. In view of the possibility of a varied response in different rabbits, all the sera to be compared were inoculated on a single rabbit. The extent of reaction was judged by the size and course of a lesion. The infective suspensions and the sera used were the same as employed in other tests and have already been described. Only filtered sera without preservative were used in these tests.

TABLE 8

TREATED ANTIGENS

Antigen	*Antigen dilutions*							*Controls*		
	50	*100*	*200*	*400*	*800*	*1600*	*3200*	*Antigen*	*Serum*	*Complement*
(1) E.b. suspension, 2-month old	3	3	3	3	1	0	0	0	0	0
(2) Supernatant of (1)	3	3	3	3	0	0	0	0		
(3) Boiled (1)	3	3	3	3	1	0	0	0		
(4) Washed e.bs. (2 washings)	3	3	3	2	0	0	0	0		
(5) Boiled (4)	3	3	3	1	0	0	0	0		

Results

The same virus suspension was used in the first three tests but a different one in the fourth (Rabbit B.75). The results (Table 9) show that normal sheep, horse and human sera did not exert any inhibitory action, nor did serum of a recovered rabbit infected 5 months previously. Convalescent and immune sera, on the other hand, showed a recognisable inhibitory effect. The number of experiments being small, this inhibitory effect was not numerically measured in terms of m.i.d. (50 per cent end-point). The absence of inhibitory action by the recovered rabbit serum in contrast to that of convalescent and immune sera is in line with the experience of infectivity tests, in which a recovered rabbit became fully susceptible after a few months.

TABLE 9
NEUTRALISATION TESTS

Test rabbit	*Virus dilution*	*Serum tested*				
D.75		*No serum*	*Healthy sheep*	*Convalescent sheep*	*Immune sheep (2 mixed)*	
					First row	Second row
	10^0	+ + +	+ + +	+ +	—	+ +
	10^{-1}	+ + +	+ + +	—	—	—
	10^{-2}	+ + +	+ + +	—	—	—
	10^{-3}	+ +	+	—	—	—
	10^{-4}	—	—	—	—	—
	10^{-5}	—	—	—	—	—
	no virus	—	—	—	—	—
D.38		*No serum*	*Recovered rabbit (5 months)*	*Convalescent rabbit (2 mixed)*	*Immune rabbit*	
	10^0	+ + +	+ + +	+ +	?	
	10^{-1}	+ + +	+ + +	—	—	
	10^{-2}	+ + +	+ +	—	—	
	10^{-3}	—	—	—	—	
	10^{-4}	+	—	—	—	
	no virus	—	—	—	—	
C.44		*No serum*	*Normal horse (1)*	*Normal horse (2)*	*Convalescent sheep*	
	10^0	+ + +	+ + +	+ + +	+	
	10^{-1}	+ + +	+ + +	+ + +	—	
	10^{-2}	+ + +	+ +	+ + +	—	
	10^{-3}	—	—	+	—	
	10^{-4}	—	—	—	—	
	no virus	—	—	—	—	
B.75		*No serum*	*Healthy human*	*Recovered human*	*Convalescent human*	
					First row	Second row
	1/50	+ + +	+ + +	+ + +	+ +	+ +
	50×10^{-1}	+ + +	+ + +	—	—	—
	50×10^{-2}	+ +	+	+	—	—
	50×10^{-3}	—	—	—	—	—
	50×10^{-4}	—	—	—	—	—
	no virus	—	—	—	—	—

DISCUSSION

By using more refined antigens than hitherto available, it was possible to show that C.P.D. virus induces the formation of agglutinating, precipitating, complement fixing and inactivating antibody (or antibodies). It does not, however, produce a haemagglutinin as do vaccinia, variola and ectromelia viruses, nor does C.P.D. antiserum inhibit vaccinia haemagglutinin.

Whereas normal rabbit and human sera showed no agglutinins, the agglutination titre of convalescent and immune sera of sheep, rabbits and humans was fairly high. This finding may be useful for the diagnosis of the disease in laboratories where there is a supply of e.bs. It appears that agglutinins are detectable in the serum of human patients after precipitins and complement fixing bodies have disappeared.

The failure of Glover (1932-33) and of Manley (1934) to detect precipitins was most probably due to the use of an unsuitable antigen. Unlike vaccinia the extract of scabs from C.P.D. either does not produce sufficient preciptinogen or contains some inhibitory substance. For this reason, the test is not of much practical value in the diagnosis of the disease.

The complement fixation tests indicated that there was a higher concentration of antibody in immune than in recovered sera. The antigen was present to an almost equal extent in the washed e.bs., in the fluid in which it was stored and in tissue extracts of early lesions. Since boiled suspensions and the supernatant fluid from centrifuged suspensions were still active, it can be concluded that the living virus is not essential.

The serologinal differentiation of vaccinia and C.P.D. by the complement fixation test, reported by MacDonald (1951), was confirmed.

CONCLUSIONS

Extracts of C.P.D. infected sheep skin and elementary body suspensions did not agglutinate fowl, pigeon, sheep, goat, horse, calf, rabbit, guinea-pig and mouse red cells, nor were human Rh-positive cells sensitised with incomplete anti-D Rh antibody or bovine red cells sensitised with the Paul-Bunnel antibody agglutinated. C.P.D. antisera did not inhibit vaccinia haemagglutinin.

Immune sera of sheep and rabbits agglutinated C.P.D. e.bs. in high dilutions and convalescent sheep, rabbit and human sera in moderately high dilutions. The use of this reaction is recommended for the laboratory diagnosis of the disease, particularly in man.

A flocculation reaction was demonstrated with C.P.D. immune sera and various extracts from e.bs. This indicated that the virus produces a soluble antigen. The technique of the vaccinia complement-fixation test was modified to demonstrate the reaction in C.P.D. The presence of living virus was not necessary for the reaction.

The presence of an inactivating antibody in immune and convalescent sera was shown by injecting the serum-virus mixtures intradermally in rabbits.

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CHANGES IN THE BLOOD PICTURE OF CALVES FOLLOWING VACCINATION WITH *BRUCELLA ABORTUS* (STRAIN 19) VACCINE

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INTRODUCTION

Linquist, Zehner and Phillips (1949) studied the effects of vaccination of young heifers with *Brucella abortus* (strain 19) and observed changes in the total leucocyte count and the percentages of the individual white cell components during the first five days following inoculation; a depressant effect on the blood plasma levels of ascorbic acid and vitamin A was also recorded. Bell and Irwin (1938) investigated the changes in the cellular constituents of the blood of cattle affected with brucellosis, while Ferguson, Irwin and Beach (1945) compared the blood pictures of healthy individuals with those obtained from the same animals when exposed to *Br. abortus* infection. Cotton (1953) vaccinated young calves 5 to 7 months of age with *Br. abortus* (strain 19) vaccine by the intracutaneous and subcutaneous routes and observed the cellular picture of the blood over a period of two years; variations in the opsonic index were also noted.

The present study was designed to determine whether similar cellular changes occur in the blood of calves vaccinated with *Br. abortus* (strain 19) vaccine when kept under ordinary farm conditions in this country and the observations were also extended to the other constituents of the blood picture. Each calf was studied for a period of 26 days following vaccination and the changes produced were correlated with the rise and fall of the serum agglutination titre. Previous work (Greatorex, 1955; Holman, 1956) has shown that the values of the various components of the blood in normal calves fluctuate with age throughout the first twelve months of life, and it was therefore necessary in interpreting the results, to ascertain whether the changes that occurred were due to the effects of vaccination or were associated with the increasing age of the animals.

MATERIALS AND METHODS

Animals. Ninety-one clinically healthy calves of different breeds and from attested herds were studied and of this number 45 were vaccinated with Strain 19, whilst the remaining 46 were left as controls. In order to take into consideration the changes which occur in the blood picture due to increasing age, the calves were classified into four groups. Group 1 containing those calves which were 4 months old at the commencement of the experiment; Group 2, 5 months; Group 3, 6 months and Group 4, 7 months. Each calf was bled at weekly intervals for four weeks. At the end of the experimental period the calves of Group 1 were 5 months old, those of Group 2, 6 months; Group 3, 7 months and Group 4, 8 months.

All the observations were made during the summer months while the calves were at pasture; hence alterations in their blood picture due to climate, seasonal variations, environment and diet were largely eliminated. The general management of the calves was excellent throughout. Both the vaccinated and control animals were bled several times before the commencement of the investigation to ascertain their normal blood picture and serum agglutination levels. Only those with a blood picture within the normal range for their age and a negative agglutination titre were included in the experimental groups during the observation period.

TABLE 1

THE DISTRIBUTION OF CALVES IN THE VARIOUS AGE GROUPS

Group	*Age of calf at commencement of experiment*	*No. of vaccinated calves*	*No. of control calves*
	months		
1	4	11	11
2	5	7	12
3	6	10	12
4	7	17	11
Total		45	46

Vaccination. Forty-five calves were vaccinated behind the shoulder with 5 ml. Strain 19 supplied by the Ministry of Agriculture, Weybridge. A close watch was kept on the calves for loss of appetite, malaise, rise of temperature and swelling at the site of inoculation.

Controls. The 46 animals which served as controls were allowed to run with the vaccinated calves.

Withdrawal of blood. Great care was exercised while taking the samples to avoid undue excitement which might bring into play the so-called "Alarm Factor" (Seyle, 1946) and give rise to abnormal recordings. Two samples of blood (5 ml.) were taken from the jugular vein at each bleeding. One of the samples, not mixed with an anticoagulant, was used for estimating the coagulation time, preparing slides for differential white cell counts and providing serum for the agglutination test. The other sample, collected into a mixture of potassium and ammonium oxalate (4:6) (0·5 ml. per 5 ml. blood), was used for carrying out the other haematological procedures. Following vaccination, blood samples were taken on the 5th, 12th, 19th and 26th days; preliminary investigations had shown these intervals to be the most appropriate for demonstrating the rise and fall in serum agglutinins.

Techniques. The techniques used for the physical and cytological examinations of the blood were identical with those already described (Greatorex, 1954).

Agglutination test. Serum from each blood sample was tested against a standardised antigen in twofold dilutions of 1 in 10 to 1 in 10,240 the agglutination reaction being recorded after 24 hours incubation at 37°C.

TABLE 2

COMPARISON OF THE HAEMATOLOGICAL CHANGES OF NORMAL CALVES AND CALVES VACCINATED WITH *BRUCELLA ABORTUS* (S19) VACCINE

Constituents	*Normal calves (Controls)*				*Vaccinated calves Days after vaccination*							
						5 Days		*12 Days*		*19 Days*		*26 Days*
	Group	*No. of animals*	*Mean*	*Range*	*No. of animals*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Coagulation time (in minutes)	1	11	5·0	3·5–7·5	11	7·9	5·0–10·5	8·4	4·0–11·0	5·8	3·0–7·5	5·0	3·5–6·5
	2	12	6·9	5·0–9·0	7	9·0	6·5–10·0	8·8	6·0–11·5	6·9	4·5–9·5	5·4	4·0–7·0
	3	12	6·8	5·5–8·5	10	9·2	8·0–10·5	9·0	7·5–11·0	6·8	5·5–9·0	6·2	4·5–8·5
	4	11	6·2	4·0–8·0	17	8·9	5·0–10·5	9·2	6·5–12·5	7·7	6·0–10·0	6·9	5·0–10·0
Erythrocyte sedimentation rate (mm)	1	11	2·0	1·0–4·0	11	3·5	1·0–6·0	3·5	1·0–5·0	3·0	2·0–4·5	3·0	2·0–4·0
	2	12	2·0	1·0–3·0	7	3·5	1·0–7·0	3·5	2·0–4·0	3·0	1·5–4·5	3·0	2·0–5·0
	3	12	2·5	1·0–5·0	10	3·5	2·0–6·0	3·5	2·5–3·5	3·0	2·0–5·0	3·0	2·5–5·0
	4	11	2·5	1·5–5·0	17	3·0	1·5–4·5	2·5	1·0–4·0	3·0	2·0–4·0	3·0	2·5–4·5
Erythrocyte count (millions per c.mm.)	1	11	7·8	6·1–12·6	11	5·6	4·6–8·9	5·6	4·0–7·7	7·1	4·9–8·2	7·5	6·5–8·9
	2	12	7·3	4·8–11·2	7	6·1	4·8–7·2	5·8	5·3–6·9	7·2	5·1–9·4	7·4	5·6–8·4
	3	12	7·3	5·2–11·1	10	6·2	5·4–7·8	5·9	4·6–7·4	7·3	4·9–7·4	7·6	6·5–10·2
	4	11	7·0	4·6–8·6	17	6·4	4·8–7·5	6·2	4·8–7·7	7·0	6·1–8·3	7·5	5·5–8·6
Packed cell volume (per cent)	1	11	43·4	34·0–52·0	11	37·3	31·5–41·5	37·1	31·0–44·0	42·8	33·0–48·0	44·2	34·0–48·0
	2	12	43·5	36·5–54·5	7	39·6	34·0–44·5	38·3	32·5–44·5	41·4	37·0–49·5	42·3	37·0–51·5
	3	12	41·2	37·0–46·5	10	35·8	31·0–39·5	34·3	30·0–43·0	38·2	34·0–48·0	40·0	36·0–47·5
	4	11	37·1	33·5–40·0	17	36·6	30·0–40·0	35·7	30·0–39·0	39·2	35·5–48·0	40·2	36·0–48·0
Haemoglobin content (g/100 ml. blood)	1	11	11·7	8·6–13·6	11	10·1	8·3–11·6	11·6	9·3–15·0	11·8	10·0–13·5	12·2	11·3–13·4
	2	12	12·4	10·7–14·4	7	11·1	8·9–13·2	11·6	9·0–11·4	11·8	10·5–13·2	12·6	11·6–14·4
	3	12	12·1	8·7–14·5	10	10·3	8·7–11·6	11·4	8·9–11·9	11·4	10·5–13·2	11·8	10·7–13·1
	4	11	10·9	9·5–12·9	17	9·6	8·7–12·8	10·6	9·3–12·8	11·1	10·1–12·5	11·6	10·7–12·8
M.C.H.C. (per cent)	1	11	28·2	22·3–34·0	11	27·3	21·7–31·4	31·1	25·0–39·5	30·3	25·9–36·3	29·0	26·0–31·9
	2	12	29·1	23·9–39·4	7	28·4	25·0–31·6	31·0	24·0–38·8	28·2	26·1–29·3	27·8	25·6–32·7
	3	12	30·4	26·0–38·2	10	28·7	25·4–35·5	31·5	27·2–38·1	30·2	26·0–33·3	28·5	24·4–35·8
	4	11	29·8	24·6–33·1	17	29·3	24·6–32·0	29·7	26·5–34·3	28·3	23·5–32·8	27·0	24·5–33·8

Constituents	Normal calves (Controls)				Vaccinated calves Days after vaccination							
						5 Days		12 Days		19 Days		26 Days	
	Group	No. of animals	Mean	Range	No. of animals	Mean	Range	Mean	Range	Mean	Range	Mean	Range
M.C.V. (cubic microns)	1	11	57·3	38·1–73·8	11	64·1	53·6–72·8	60·7	48·0–77·5	56·6	43·6–70·0	53·0	45·7–68·5
	2	12	59·9	37·9–92·7	7	65·9	54·8–92·7	61·3	49·2–83·9	60·6	39·3–87·0	58·4	46·3–78·5
	3	12	54·8	38·4–89·4	10	55·4	47·7–72·2	55·1	40·5–86·9	54·4	39·4–73·4	53·2	38·7–57·5
	4	11	50·1	41·3–68·2	17	57·0	45·7–72·6	56·7	47·7–92·6	55·7	45·1–71·7	53·1	41·8–70·3
Total leucocyte count (10^3 per c.mm.)	1	11	9·9	7·5–10·3	11	11·0	8·3–12·5	11·4	9·8–13·3	10·4	7·5–12·2	10·9	8·1–12·3
	2	12	9·6	6·0–9·8	7	10·0	9·0–11·0	10·6	9·9–11·8	9·2	6·2–11·0	9·7	8·8–11·6
	3	12	9·7	7·2–10·3	10	10·9	9·2–12·9	11·4	9·7–13·4	10·5	9·2–12·2	10·9	8·7–11·3
	4	11	9·8	6·2–10·5	17	11·4	9·7–13·5	11·9	9·9–14·6	10·2	8·6–12·2	10·8	9·2–12·4
Neutrophil count. (per cent)	1	11	19	10–34	11	28	24–36	31	21–38	20	13–34	24	12–28
	2	12	20	10–35	7	27	21–34	29	25–34	17	11–24	26	17–32
	3	12	23	12–31	10	27	21–33	30	26–34	17	13–20	26	20–34
	4	11	22	6–30	17	26	20–41	29	21–41	17	12–20	24	20–28
Lymphocyte count. (per cent)	1	11	69	49–78	11	65	49–75	63	56–73	70	56–83	68	61–77
	2	12	69	61–81	7	69	59–77	66	61–71	79	67–87	71	61–80
	3	12	63	55–74	10	67	57–73	65	60–72	76	68–84	66	49–77
	4	11	67	49–82	17	71	53–79	68	56–76	79	62–85	73	68–78
Eosinophil count. (per cent)	1	11	5	2–8	11	4	0–9	4	0–10	6	0–12	6	0–13
	2	12	5	1–8	7	2	1–4	3	0–11	3	0–8	1	0–4
	3	12	8	1–12	10	4	0–8	3	1–6	5	1–11	4	0–11
	4	11	6	2–13	17	2	0–4	1	0–4	1	0–5	2	0–6
Monocyte count. (per cent)	1	11	7	2–12	11	3	0–6	3	0–5	3	1–6	3	0–5
	2	12	9	3–12	7	1	1–3	2	0–5	2	0–7	2	0–4
	3	12	5	0–9	10	3	0–5	3	1–4	2	0–4	3	0–7
	4	11	5	1–12	17	2	0–4	1	0–4	1	0–5	2	0–6

Group 1 4–5 months. Group 2 5–6 months. Group 3 6–7 months. Group 4 7–8 months.

Agglutination at 1:10 was regarded as negative, 1:20 indefinite and above 1:20 positive.

RESULTS

In only a few calves were deviations from normal health observed, during which time no significant changes could be detected in the blood pictures. Those calves which did show slight malaise had recovered from the effects of the inoculation by the 2nd or 3rd day. Although all the calves did not respond in exactly the same way following vaccination, definite trends could be detected in the various blood constituents in 85 per cent. In 7 per cent the response to vaccination was delayed for a few days, but the same sequence of events could be observed. The blood changes in the remaining 8 per cent showed such marked variation that the results could not be interpreted. The mean values and ranges obtained for vaccinated and control calves of the four age groups studied are shown in Table 2.

Agglutination Tests

Detectable levels of agglutinins appeared in the serum by the 5th day following vaccination and two thirds of the calves showed maximum titres by the 12th day. They remained at this level for a week and then gradually began to wane from about the 19th day. By the end of the 26th day the majority of calves were showing either a low positive titre or an indefinite reaction. Maximum titres were generally between 1 : 80 and 1 : 640, but a few calves developed titres of between 1 : 640 and 1 : 5120. In one case a titre of 1 : 10,240 was reached by the 12th day following vaccination. These high titres did not appear to have any additional influence upon the blood picture.

Examination of Blood

Coagulation time. Corresponding with the initial rise in the agglutination titre, the coagulation time for many of the vaccinated calves of the four age groups became prolonged by 3 to 4 minutes as compared with that of the controls. This delay persisted while the titres were at their maximum, but coinciding with the fall in the agglutination titre at the 19th day the blood coagulation became more rapid and by the 26th day values approximating those of non-vaccinated calves were reached.

Erythrocyte sedimentation rate. In man the erythrocyte sedimentation rate depends to a large extent upon rouleaux formation of the red cells, a property which is influenced by the concentration of fibrinogen and other proteins in the plasma. This formation does not occur in bovines and, as the suspension stability of red cells is great, the rate of sedimentation in the bovine is not a true one. Ferrando (1937) has stated that in cattle the E.S.R. is of little significance and must be measured within a few hours of collecting the blood. By allowing the blood to stand in the vertical position for

24 hours before taking readings, it was found that by the 5th and 12th days following vaccination, i.e. while the agglutinins were forming and reaching their maximum titre, the rate of sedimentation was increased, in many cases by as much as 5 mm., particularly in Groups 1 and 2 (4 to 5 and 5 to 6 month old calves). These increased values persisted while the agglutinins were waning and the return to normal values was delayed for some time, which suggests that the presence of agglutinins affects in some way the stability of erythrocytes. A comparison of the curves drawn for the erythrocyte sedimentation rate and for the haemoglobin content showed a close similarity, but no correlation between these two values could be deduced.

Fragility of erythrocytes. No significant alterations in the fragility of erythrocytes were observed following vaccination.

Platelet count. As the counting of blood platelets is subject to considerable technical errors, the marked variations in the recordings were assumed to be due to experimental error rather than the effects of vaccination.

Erythrocyte picture. By the fifth day following vaccination, when the serum agglutinins were becoming apparent, the erythrocyte count, packed cell volume and haemoglobin content of the blood of the calves in the four age groups showed significantly reduced values compared to those of non-vaccinated calves of the same age. This downward trend continued in the case of the erythrocyte count and the packed cell volume, until titres were maximal on the 12th day. Subsequently the values for these two components increased to become normal by the 26th day when titres were either indefinite or negative. The reduction in haemoglobin appeared to be transient, for at the time titres were at their peak normal haemoglobin values were recorded and remained comparable to those of normal calves. The erythrocyte picture of normal calves during the same period showed a gradual fall.

The mean corpuscular haemoglobin concentration (M.C.H.C.) increased between the 5th and 12th day, when agglutinins were attaining their maximum. Mean corpuscular volumes (M.C.V.) increased during the immediate post-vaccination period but returned to normal by the 26th day.

Leucocyte Picture

Total leucocyte count. A close correlation was found between the total leucocyte count and the formation and subsequent decline in agglutinins. A gradual increase in the total number occurred as the agglutinins appeared; this response was particularly noticeable in calves of the first and fourth age groups (4 to 5 and 7 to 8 months). As the titres commenced to decline between the 12th and 19th day, definite reductions in the number of leucocytes occurred. In the majority of the vaccinated calves an additional response was

observed when titres were approaching an indefinite or negative reaction, but this appeared to be of a secondary nature. Cotton (1953) also recorded this secondary increase, but not until the second month after vaccination.

Differential Count

Neutrophils. The changes in the percentages of neutrophils were essentially the same as those described for the total leucocyte count, indicating that the rise was due to an increase in the number of circulating neutrophils. As noted with the total leucocyte count, a temporary secondary response occurred at the time the titres were indefinite or had become negative. Only two calves showed any increase in "band" forms following vaccination.

Lymphocytes. As the percentage of lymphocytes in the bovine is inversely proportional to that of neutrophils, a reversal of the picture described for the total leucocyte and neutrophil counts was obtained. Thus in the immediate post-vaccination period a marked reduction occurred in the percentage of lymphocytes with a gradual rise to pre-vaccination levels following the attainment of maximum titres. Corresponding with the temporary rise in neutrophils, reduced percentages of lymphocytes were obtained.

Monocytes. Vaccination resulted in an increase in the percentage of monocytes in the majority of calves, these increased values being maintained throughout the period of observation. It would seem that once monocytic stimulation has been induced a considerable delay occurs before the return to normal values.

Eosinophils. Although no significant variations occurred in the eosinophil percentages, some calves showed an eosinophilia which could not be attributed to other factors e.g. parasitic infestations; this eosinophilia invariably coincided with the disappearance of agglutinins.

DISCUSSION

The evidence presented indicates that changes occur in the blood picture of calves following vaccination with *Br. abortus* (strain 19) vaccine. When compared with normal calves of the same age, 85 per cent showed the changes described and these occurred irrespective of those attributable to the increase in the age of the calves. The remaining calves either failed to show any distinctive changes in the blood picture or showed alterations only in accordance with their increasing age. Previous work has been confined to the variations in the erythrocyte and leucocyte pictures, but in this survey consideration was also given to other components of the blood picture.

The coagulation time of blood and, to a lesser extent, the erythrocyte sedimentation rate, appear to be influenced during the formation and disappearance of agglutinins, but no significant

alterations in the fragility of erythrocytes could be detected and no definite trends were observed in the platelet counts.

The changes recorded for the erythrocyte picture were not always in agreement with those described by other authors. Cotton (1953) found that the erythrocyte counts varied so little following vaccination that they were not considered significant. Ferguson, *et al.* (1954) recorded decreases in the number of erythrocytes in cattle following artificial infection with *Br. abortus*. The vaccinated calves of our series showed a similar reduction in the immediate post-vaccination period i.e. during the formation of agglutinins, but subsequently the erythrocyte values gradually increased as the titres began to fall, until they approximated those of normal calves. Packed cell volumes showed a similar trend.

Ferguson *et al.* (1945) also recorded a decrease in the average number of leucocytes in the blood of cattle following infection. In vaccinated animals, however, most observers have noted increased values. Lindquist *et al.* (1949), who confined their observations to the first five days following vaccination, recorded a distinct rise in the leucocyte count on the first day with a return to normal by the fifth day. Cotton (1953), found that although the leucocyte count showed an increase on the first day following vaccination, it had fallen to below the normal by the fourth day and was still lower by the eighth. In our investigation the leucocyte reponse lasted until the maximum titres had been reached, about the 12th day, and was followed by a reduction in the values as the titres waned. A temporary leucocyte response when the titres become indefinite was also noticed by Cotton (1953), but was delayed until the second month after vaccination. This secondary response was due to an increase in the number of neutrophils, but there was no evidence of a "shift to the left," as "young" and "band" forms of neutrophils were scarce during the phase of leucocytosis.

Vaccination was followed by a reduction in the number of lymphocytes until maximum titres were recorded, followed by an increase. This is in agreement with the findings of Ferguson *et al.* (1945) for artificially infected animals, and for the vaccinated animals of Lindquist *et al.* (1949). Cotton (1953) found a considerable increase in the number of lymphocytes by the fourth day, counts up to 95 per cent being recorded for some individuals. This lymphocytosis persisted for 7 to 8 days after which the percentages returned to normal. Although Cotton found little or no variation in monocyte percentages, the calves in our series showed increased values, which persisted throughout the period of observation.

No significant variation in the number of eosinophils was observed, although an eosinophilia of varying degree could be detected in a few calves as the titres disappeared. Cotton noticed a similar increase two months after vaccination.

Previous work (Greatorex, 1955; Holman, 1956) has shown that age influences the blood picture of calves during their first year of

life. The former author has also indicated that certain changes occurring in calves of 5 to 9 months of age may be due to the onset of puberty or to changes in the rate of growth; these changes consisted of prolonged clotting times, increased values for the components of the erythrocyte picture and higher eosinophil and monocyte percentages. In assessing the results, these other factors have been taken into account and it is concluded that the changes recorded, occuring in 85 per cent of the calves studied, were due to the effects of vaccination with *Brucella abortus* (Strain 19) vaccine.

CONCLUSION

The changes in the blood pictures of calves of 4 to 8 months of age following vaccination with *Brucella abortus* (Strain 19) vaccine have been described and compared with those of normal calves of the same age and kept under identical conditions of animal husbandry. The changes noted have been correlated with the rise and fall of agglutination titres.

In the assessment of the results, account has been taken of the variations in the blood picture associated with increasing age, puberty and growth of the calves.

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THE INTRAVENOUS INOCULATION OF SHEEP WITH GRADED DOSES OF *MYCOBACTERIUM JOHNEI*

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INTRODUCTION

The immunity of cattle and sheep vaccinated against Johne's disease has been and is being assessed on field results, but the collection of data is difficult and a comparison of the immunising properties of different vaccines in the field has not been attempted. Live vaccines of varying degrees of virulence and dead vaccines are used but there is no information on their comparative merits, nor is it likely that critical information will become available until such time as the resistance of vaccinated animals can be assessed by exposure to a measured weight of infection. The preliminary step towards this goal is the determination of a dose of *Mycobacterium johnei*, wherewith to test the resistance of the vaccinated animal, which is of adequate infectivity but not one that would overwhelm the degree of resistance that might be hoped for from vaccination. The experiments reported in this paper were undertaken with this end in view.

METHODS AND MATERIALS

Experimental animals. Eighty-one six months old Blackface lambs born at the Institute were used; so far as was known they had not been exposed to infection with *M. johnei.*

The lambs were divided into five groups, four containing sixteen lambs, and the fifth seventeen lambs. The lambs in the first four groups were each inoculated intravenously in October, 1954, with a culture of *M. johnei*, the dose being different for each group. The seventeen lambs in the fifth group were kept as uninoculated controls. The animals, two from each group per pen, were housed in eight separate covered pens with cement floors and walls. The seventeenth sheep in the control group was added to pen 8, bringing the number of uninoculated sheep in this pen to three. They remained in their pens until March, 1956, when the survivors were turned out to pasture and ran as a flock for eight weeks. Thereafter they were returned to and remained in their respective pens until slaughtered. The sheep while housed were fed hay and a small ration of concentrates. The pens were without bedding, and were swept out daily except on Sundays.

Culture of M. johnei *inoculated.* The second sub-culture of a recently isolated bovine strain grown on Dubos medium as modified by Williams Smith (1953) for six weeks, was washed off with sterile distilled water and reduced to a macroscopically homogenous suspension by grinding in a Griffith tube. Microscopically the suspension contained single bacilli and small clumps of variable numbers of bacilli. The suspension was adjusted to tube 2 of Brown's scale; one ml. of the adjusted suspension

contained approximately 2 mg. of wet bacilli. Dilutions of the suspension of 10^{-2}, 10^{-3} and 10^{-4} were made with distilled water. The dose of the suspension and of each dilution was 1 ml. inoculated intravenously on the 11th of October, 1954.

Examination of the sheep for the presence of M. johnei *in their tissues.* Selected lymph glands were collected with sterile precautions and cultured. Each gland when small, or multiple portions of larger glands, was minced. A small quantity of sterile distilled water was added and the material ground in a Griffith tube; oxalic acid was added and the material given a further grinding before filtering through muslin. The filtered material, after incubation at 37°C. for fifteen minutes, was centrifuged and the deposit taken up in sufficient distilled water to provide inocula for six slopes of modified Dubos medium. The final examination of the cultures was made after not less than three months incubation.

Haemolysis test. Before inoculation and at intervals up to July 1956, the sheep were bled and their sera tested by the haemolytic modification of the haemagglutination test (Middlebrook, 1950). Fisher and Gregory (1951) have suggested that a haemolytic test might detect individual animals infected with *M. johnei.*

Sensitization of sheep's red blood cells. Blood from the jugular vein of a sheep, collected in an equal volume of sterile Alsever's solution, was allowed to stand for 24 hours at 4°C. and was discarded after 10 days. The cells were washed three times in normal saline, and packed; 0·4 ml. of packed cells was added to 10 ml. of buffered saline (pH 7·1) to which 0·1 ml. of mammalian P.P.D. tuberculin (1 mg. P.P.D.) had been added. The cells were incubated in the diluted tuberculin at 37°C. for 2 hours, shaking at 15 minute intervals; they were then washed once, the supernatant discarded and the cells resuspended in buffered saline to give an 0·5 per cent suspension. The sensitized cells may be kept at 4°C. up to 3 days. (In preliminary tests mammalian and avian p.p.d. tuberculins gave comparable results with known sera, and since more of the former was available, it was used).

Test sera. These were inactivated at 56°C. for 30 minutes and 1.5 ml. of a 1/5 dilution was absorbed at room temperature for 15 minutes with 0·2 ml. of packed normal red blood cells. After centrifuging, the supernatant serum was used as a 1/5 dilution.

Complement. Preserved guinea-pig complement (B.W. & Co.) was diluted 1/4 with distilled water and absorbed at 4°C. for 15 minutes with a volume of packed normal red blood cells equal to the volume of neat complement used. The cells were then packed and the supernatant complement diluted with a further 1·5 volume of distilled water and this 1/10 dilution used in the test proper.

Throughout doubling dilutions of the test sera in 0·2 ml. volumes were made in buffered saline (pH 7·1) and equal volumes of sensitized cells added. These were incubated in a water bath at 37°C. for 1 hour, the racks being shaken every 20 minutes; 0·2 ml. of 1/10 complement was then added, the tubes well shaken and incubated for a further 30 minutes when readings were made.

As controls known positive and negative control sera kept at −20°C. in suitable aliquots, were included in each test, and used throughout the experiment. A 1/10 dilution of each serum was tested against unsensitized sheep cells.

Tuberculin test. The reactions of the sheep to the intradermal injection of avian tuberculin were tested from time to time.

RESULTS

Post-mortem and Cultural Findings

One year after inoculation, all the sheep having remained healthy, one of those inoculated with the undiluted suspension was slaughtered. No lesions were found, but *M. johnei* was cultured from the caecal, portal and the left internal iliac lymph glands. As soon as evidence of growth was obtained further sheep were slaughtered at the rate of about two per week until by February, 1956, thirty had been examined. None showed lesions.

Meantime two sheep in pen 8 inoculated with undiluted culture developed clinical Johne's disease and when *M. johnei* was found in the faeces they were slaughtered, the first in January, 1956, and the second a month later. Thereafter no further clinical cases of disease occurred. From the end of May, 1956, except during August, two to three of the remaining forty-nine sheep were slaughtered and examined each week until by mid-October 1956, all were disposed of. The three control sheep in pen 8 were among those slaughtered in October.

The results of the cultural examination of lymph glands from all the sheep are summarised in Table 1. The lymph glands cultured from the sheep slaughtered up to February 1956 were: three individual mesenteric glands, caecal, portal, posterior mediastinal, right bronchial, left bronchial, right internal iliac, left internal iliac, the combined prescapular and the combined precrural glands. From the remaining forty-nine sheep five individual mesenteric glands were cultured, or where five separate glands were not available the number was made up by using portions of large glands; the caecal, colic, portal, mediastinal, right internal iliac and left internal iliac lymph glands were also cultured.

M. johnei was recovered from three uninoculated control sheep, all in pen 8 that housed the two clinical cases and proven excretors of *M. johnei*. It is unlikely that these control sheep were infected when they entered the experiment, but highly probable that they acquired the infection between December 1955 and early February 1956, when twenty or more months of age, from the ingestion of *M. johnei* excreted by the two clinical cases. When the control uninoculated sheep were slaughtered, they had probably been infected for about ten months. The weight of the evidence is in favour of the *M. johnei* recovered from all but the three controls stemming directly from the inoculations. There may, however, be five possible exceptions, namely the five inoculated sheep in pen 8 found infected. Two of these had been inoculated with the 10^{-2}, two with 10^{-4} and one with the 10^{-6} dilutions of the suspension of *M. johnei*.

Table 2 shows the frequency of the cultivation of *M. johnei* from

TABLE 1

THE SUMMARISED RESULTS OF THE CULTURAL EXAMINATIONS OF LYMPH GLANDS

Number of sheep and interval between date of inoculation and slaughter	*Infective dose*								*Controls uninoculated*	
	Undiluted		*Dilution* 10^{-3}		*Dilution* 10^{-4}		*Dilution* 10^{-6}			
	Positive	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*
30 12-16 months	5	1	6		4	2	3	3		6
2 (clinical Johne's disease) 14-15 months	2									
49 19-24 months	7	1	6	4	8	2	2	8	3*	8
Total 81	14	2	12	4	12	4	5	11	3	14

* The three sheep were in contact with the two clinical cases of Johne's disease.

TABLE 2

THE NUMBER OF TIMES THE DIFFERENT LYMPH GLANDS EXAMINED WERE FOUND INFECTED

Number of infected sheep and interval between inoculation and slaughter	*mesenteric*	*caecal*	*portal*	*mediastinal*	*right internal iliac*	*left internal iliac*	*colic*	*right bronchial*	*left bronchial*	*prescapulars*	*precrurals*
18 12-16 months	9	12	11	0	1	1	not examined	0	0	0	0
2 (clinical disease) 14-15 months	2	2	2	2	2	1	not examined	2	1	1	1
23 19-24 months	19	12	11	3	0	0	8	not examined			

the different lymph glands of the inoculated sheep. Those most frequently infected were the intestinal and the hepatic glands. In the five instances where the mediastinal gland was infected the lymph glands of the intestine and the portal lymph gland were also infected. Infection of the bronchial glands, the prescapular and the precrural lymph glands occurred in the two clinical cases but in no other animal from which these glands were examined.

The mesenteric, caecal, portal, mediastinal and internal iliac lymph glands were examined from every sheep and in every infected sheep *M. johnei* was cultured from one or more. When a score of one was given for infection for each of the above individual glands, or group of glands, the infectivity scores for the groups of sheep inoculated with the different doses of *M. johnei* were as follows:— undiluted suspension, 36; dilution 10^{-2}, 25; dilution 10^{-4}, 20; dilution 10^{-6}, 8. These infectivity scores together with the results presented in Table 1 indicate that the graded doses produced degrees of infection in ordered sequence.

Haemolytic and Skin Sensitivity Tests

Before the inoculation of *M. johnei* no positive reactors to either the haemolytic test or the skin sensitivity test were found. Subsequently ten flock tests were made, the first in December, 1954, and the last in July, 1956. The titres of the sera showing complete haemolysis ranged from 1/10 to 1/640, partial haemolysis was given by one serum at 1/2560. The results of the tests may be summarised as follows :—

Sixteen sheep inoculated with undiluted suspension (*2 mg. wet bacilli*). Thirteen reacted at the first test. At subsequent tests all but two reacted consistently. One of the latter developed clinical disease. Its serum was negative at the first test but had a titre of 1/10 at the second test; thereafter it was negative for four consecutive tests, but at the seventh test, seven weeks before slaughter, it gave partial haemolysis at 1/40. The serum from the second sheep started by giving a titre of 1/160, but at six consecutive tests, before slaughter, it was negative. This animal although not a clinical case had nevertheless heavily infected lymph glands.

Sixteen sheep inoculated with dilution 10^{-2}. Eleven sheep gave inconsistent reactions and there was little correlation between a positive or negative reaction and the presence or absence of infection of the lymph glands.

Sixteen sheep inoculated with dilution 10^{-4}. Five sheep in this group gave irregular reactions at a low titre. Eight of the animals that never reacted were found infected.

Sixteen sheep inoculated with dilution 10^{-6}. None gave a reaction but five were found to have infected lymph glands.

Seventeen control non-inoculated sheep. One sheep gave a positive reaction. At each of the last five of the eight tests made the titres

ranged from 1/40 with a progressive increase to 1/160. *M. johnei* was not recovered from this animal slaughtered two months after showing a 1/160 titre. Of the three animals that became infected one never reacted, the second gave partial haemolysis at titres of 1/10, 1/20 and 1/10 at the first, second and fourth test. At the ensuing six tests it was negative. The third sheep gave negative reactions at the first six tests and at the seventh and ninth tests, but at the eighth and tenth tests showed titres of 1/20 and 1/40. On all three infected sheep five tests were made subsequent to exposure to *M. johnei* excreted by in-contact animals. These results show that the test as used was valueless in the critical detection of infected animals.

With regard to the skin sensitivity test more positive reactors were found in the sheep that received the two larger inoculations of *M. johnei* than in the other inoculated animals, but a positive reaction or a series of positive reactions was sometimes followed by failure of animals, later found to be infected, to react. On the other hand some sheep that reacted were not found to be infected. These inconsistencies made the test valueless.

DISCUSSION

The immunity of sheep to *M. johnei* might be measured if following an intravenous test inoculation of the bacilli one or more of the following criteria were fulfilled: (1) the prevention of the characteristic macroscopic lesions and the appearance of symptoms in the immune animal; (2) the destruction of the bacilli in the tissues of the immune animal; (3) the limitation of the spread and possibly the multiplication of the bacilli in the immune animal. In the experiments reported in this paper the selection of six months old lambs was governed by the following considerations. At three to four months of age lambs should have developed the capacity to react satisfactorily to an immunological stimulus. The immunity of animals vaccinated at that age might therefore be tested some weeks later, the lambs then being about six months old and of an age when the supposed age resistance to infection with *M. johnei* might not yet be fully operative.

If immunity can prevent the development of lesions or symptoms its demonstration would be relatively simple, although a test inoculation greater than the largest used in the present series of experiments on normal animals would be required. If, on the other hand, immunity to *M. johnei* is only of a low grade a test inoculation that causes macroscopic lesions and clinical disease in normal animals might overwhelm and mask the immunity of a vaccinated animal.

In those animals found infected, but without macroscopic lesions, the mediastinal lymph gland was seldom infected, the internal iliac lymph glands were infected but once, and the bronchial lymph glands and the prescapular and precrural lymph glands were

not infected. These results suggest that the tissues drained by the above mentioned lymph glands offer an unfavourable environment to *M. johnei*. Furthermore, they demonstrate the improbability of lymph glands becoming infected by bacilli reaching them in their blood supply. Conversely, the frequent recovery of the bacilli from the lymph glands draining the intestine is compatible with a susceptibility of the intestinal tissues to *M. johnei* and probably with an unrecognised multiplication of the bacilli in these tissues, which might afford a means for the demonstration of immunity, provided that in the immune animal the bacilli are destroyed in these tissues or their multiplication inhibited and their invasion of the lymph stream and lymph glands prevented or measurably delayed.

Even when the test dose is small the destruction of the bacilli in the intestine or the associated lymph glands of the vaccinated and hypothetical immune animal is problematical. The recovery of the bacilli from the regional lymph glands of the intestine up to two years after inoculation is not encouraging. In those animals found infected the numbers of colonies in cultures from the lymph glands varied from animal to animal irrespective of the interval between inoculation and slaughter, and any immunity stimulated by the bacilli in the body played no obvious part in their destruction during the second year following inoculation. Whether in the immune animal the bacilli may be destroyed or their multiplication delayed or arrested, and consequently their invasion of the regional lymph glands prevented, delayed or overcome, can only be determined by experimentation. In such experiments it is doubtful whether the information given by cultural methods would be supplemented by histological studies. Failure to find microscopic lesions in arbitrarily selected portions of the intestine would be no proof that lesions were not present, and an attempted comparison of the microscopic lesions in vaccinated and control animals would probably be misleading.

The liver is not regarded as a predilection site for *M. johnei* but the ability of the liver to remove foreign particulate matter from the blood stream might account for finding the portal lymph gland commonly infected. The possibility of an accelerated destruction of the bacilli in the liver of the immune animal, irrespective of whether or not the portal lymph gland is infected, should not be lost sight of, and experiments on immunity should be designed to take into account this possibility, making use of both cultural and histological techniques. Information is not available on whether or not the portal lymph gland of the sheep receives lymph from the proximal portion of the small intestine. Where five mesenteric lymph glands were examined culturally the recovery of *M. johnei* from the most proximal of these glands was exceptional since, out of nineteen inoculated sheep with infected mesenteric lymph glands, the most proximal gland was found infected three times only. It has therefore been assumed that *M. johnei* reached the portal lymph gland via the liver.

The readiness with which oral infection may occur is shown by the three control non-inoculated animals which became infected when approximately twenty months of age. In each of these sheep the infection was confined to a single mesenteric lymph gland and cultures from two of these provided innumerable colonies indicating an active multiplication of the bacilli in the tissues. These three sheep show that the mature animal may still be susceptible, a fortunate finding because if it were otherwise sheep would surely be useless animals for experiments on the duration of immunity.

CONCLUSIONS

Four groups of 16 six months old sheep were inoculated intravenously with graded doses of a culture of a bovine strain of *M. johnei*. There was an uninoculated group of 17 sheep.

From one to two years after inoculation the sheep were slaughtered and selected lymph glands were cultured for evidence of infection with *M. johnei*.

A dose of 2 mg. infected fourteen of sixteen sheep, two becoming clinical cases. Dilutions of 10^{-2} and 10^{-4} each infected twelve of sixteen sheep; a dilution of 10^{-6} infected five of sixteen sheep; none of the sheep developed a clinical disease. Two clinical cases occurred in a pen that housed three non-inoculated sheep: the latter were found infected at slaughter. None of the remaining fourteen control sheep kept in contact with inoculated animals that showed no clinical disease became infected.

It is considered that the three non-inoculated but infected sheep acquired infection, when about twenty months old, from the bacilli excreted in the faeces of the two clinical cases.

A serological test using as antigen sheep's red blood cells sensitized with P.P.D. tuberculin detected few infected sheep and as used was of little or no diagnostic value.

ACKNOWLEDGMENT

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SOME ASPECTS OF THE METABOLISM OF 4:6-DINITRO-*O*-CRESOL (DNC) BY THE RUMINANT

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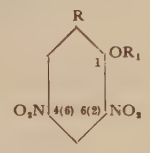
INTRODUCTION

DNC is the most important member of a group of substances that are derived from dinitrophenol. Their main uses are for controlling weeds that interfere with growing cereals (DNC), with peas (DNBP) and in winter washes for fruit trees. DNC has also been employed with some success in countering locust invasions in tropical and sub-tropical countries.

It has been reported that in 1950 at least 300,000 acres in the United Kingdom were treated with DNC and similar compounds (Toxic Chemicals in Agriculture, 1951); it is now fairly certain that this acreage has expanded considerably, thereby increasing the risks to man and to animals.

Basically DNC is a substituted dinitrophenol. It is marketed in various formulations and described by trivial names that give some indication of other groups of the basic molecule. Some of the more common derivatives are given in Table 1.



2:4 dinitrophenol (numbering clockwise from OH)

or

4:6 dinitrophenol (numbering anti-clockwise when there are other substitutents of the nucleus)

These compounds are applied by sprays in aqueous suspensions and solutions, usually as the alkaline salts, but occasionally the *iso*propanolamine and triethanolamine salts of the 'free' nitrophenols may be employed.

All DNC type compounds are toxic to man and to animals, and very strict safety measures must be adopted to prevent human casualties (Toxic Chemicals in Agriculture, 1951). While much information exists on the effects of DNC on man (Bidstrup and Payne, 1951; Harvey, Bidstrup and Bonnell, 1951; Bidstrup, Bonnell and Harvey, 1952), on laboratory animals (Parker, Barnes and Denz, 1951; King and Harvey, 1953 a, 1953 b), and on wild life (Toxic Chemicals in Agriculture, 1955), little is known about risks to the larger farm animals. The only reported case of suspected DNC poisoning is that of Edson and Fenwick (1955) who investigated the deaths of some Friesian cattle supposedly caused by DNC. While it was possible that the animals had been exposed to DNC, there was no real evidence that it had caused their deaths.

It is generally accepted that one of the most important routine measures that must be adopted in protecting men exposed to DNC is to carry out periodic blood DNC estimations. This can give unequivocal evidence of exposure and absorption, and the values obtained may be correlated with symptoms of poisoning. There seems to be no reason why this simple check should not be applied to farm animals (Edson and Fenwick, 1955).

TABLE 1

Nature of group	*Chemical name(s)*	*Trade name or initials*
R = H R_1 = H	2:4-dinitrophenol	DNP
R = Methyl R_1 = H, Na, NH_4	'Free', Na or NH_4 salt of 4:6-dinitro-*o*-cresol, *or* 2-methyl-4:6-dinitro-*o*-phenol	DNC DNOC Denocate
R = *Secy*-butyl R_1 = as for DNC	'Free', Na or NH_4 salt of 2-*secy* butyl-4:6-dinitrophenol	DNBP Dinoseb
R = *cyclo*hexyl R_1 = as for DNC	'Free', Na or NH_4 salt of 2-cyclohexyl-4:6-dinitrophenol	Dinex

In order to facilitate the practical application of this test simple experiments have been designed to supply basic information on two separate but closely related problems, the effect of isolated rumen contents on DNC, and the blood DNC levels in ruminants resulting from single doses administered orally and by intraperitoneal injection.

EXPERIMENTAL METHODS

Effect of DNC on rumen contents. Rumen contents were obtained from a goat via a rumen fistula. The animal was kept in a stall and was supplied with normal fodder. The rumen contents were filtered through coarse muslin before use in order to remove most of the larger particles of straw etc. An aqueous solution of the sodium salt of DNC (0·1 per cent, w/v) was added in appropriate amounts to 10·0 ml quantities of the filtered rumen fluid, and the final volume made up to 12·5 ml. with saline. Besides the 'blank', five tubes were prepared giving initial concentrations of 0·1, 0·2, 0·3, 0·4, 0·5 mgm DNC/ml. respectively. All additions, extractions etc. were carried out at 37 to 40°C. DNC was estimated in the tubes at 0·5 and 1·0 hours after its addition.

Administration of DNC to animals. A goat (30 kg), 2 castrated bull calves (95, 100 kg), and eight Blackface crossbred sheep (65 to 75 kg) were used as experimental animals. With the exception of six of the sheep that were kept under pasture conditions, the remaining animals were maintained in stalls and fed normal winter rations. DNC was admini-

stered orally as the sodium salt in gelatine capsules, or as a 5·0 per cent aqueous solution by intraperitoneal injection.

Scheme of dosing. For two of the sheep and the calves the following procedure was adopted :—

Animal	*First day*	*Second day*	*Third day*
1	1 mg./kg. I.P.	Rest	10 mg./kg. I.P.
2	10 mg./kg. Oral	Rest	50 mg./kg. Oral

Three doses were administered to the goat on three consecutive days.

Of the six remaining sheep four were given a single dose of 20 mg./kg. as the dry sodium salt of DNC, and each of the other two a single 10 mg./kg. dose by intraperitoneal injection.

Blood samples. These were taken from the jugular vein at 0·5 1·0, 2·0, 4·0, 6·0 and 24·0 hours after dosing (the goat, two of the sheep and the two calves). For the remainder of the sheep one sample was taken from each animal daily for five days. Some intermediate samples were also taken and also at periods greater than 24 hours after dosing.

Analysis of DNC in blood and in rumen contents. The method adopted was based on the technique of Parker (1949) modified by Edson and Fenwick (1955). This eliminates interference by carotene which is found in significantly high concentrations in the blood of some ruminants.

Method. Blood (2·0 ml.) was added to saline (1·0 ml., 0·85 per cent w/v), in an ordinary tapered centrifuge tube. Hydrochloric acid (1 ml., 5N) was added and after vigorous shaking, chloroform (2.5 ml.). The mixture was again shaken and centrifuged (5 to 10 min., 2 to 3,000 r.p.m.). The chloroform extract was removed by a pasteur pipette and exactly 2.0 ml. placed in a graduated centrifuge tube. Aqueous sodium carbonate (5.0 ml., 5·0 per cent w/v) was added, the mixture was shaken vigorously and centrifuged (2 to 3 min., 1,000 r.p.m.). An aliquot (4·0 ml.) of the aqueous supernatant layer was added to methyl ethyl ketone (4.0 ml.), followed by excess salt mixture (Parker, 1949). This was well shaken and the density of the colour of the separated ketone extract measured at $\lambda = 430$ mμ on a Hilger, 'Spekker' absorptiometer) in the usual way. While this method proved to be entirely satisfactory for bovine blood, occasional samples of rumen fluids were heavily pigmented and some of this pigment was carried through to the final extract. The results given, however, were obtained from rumen contents that gave good 'blank' values when subjected to the described technique.

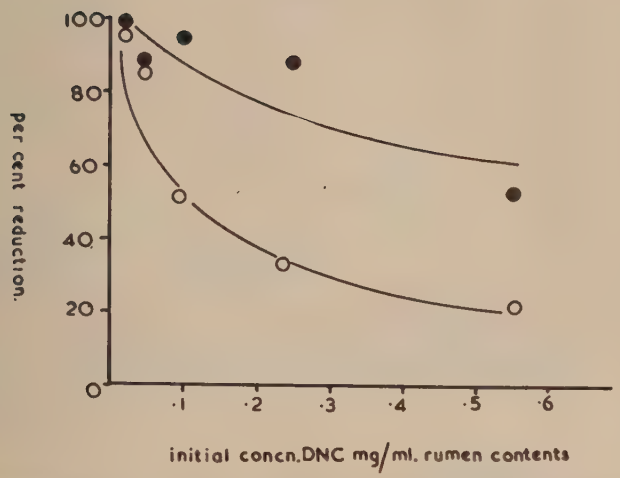
RESULTS

The results are set out in Figs 1 to 6 and Table 2.

Detoxicating Action of Rumen Contents on DNC.

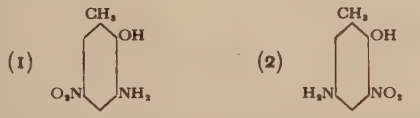
Concentrations of DNC less than 0·1 mg/ml. are rapidly and completely destroyed by rumen contents in the course of 0·5 to 1·0 hours (Fig. 1). Higher concentrations are less rapidly although still significantly decreased. While the end products of the reaction have

Fig. 1



Detoxicating action of rumen contents on DNC. The DNC concentrations along the abscissa are *initial* concentrations, and the ordinate values are the percentage reductions of each concentration after 0·5 hr. o——o and 1·0 hr. ●——●

not yet been identified, a lead as to their possible identity was provided by the fact that in the lower concentrations the original green-yellow colour of the mixture changed rapidly to a dark reddish brown. This suggested that one or other of the nitro-groups was reduced to an amino group thus:

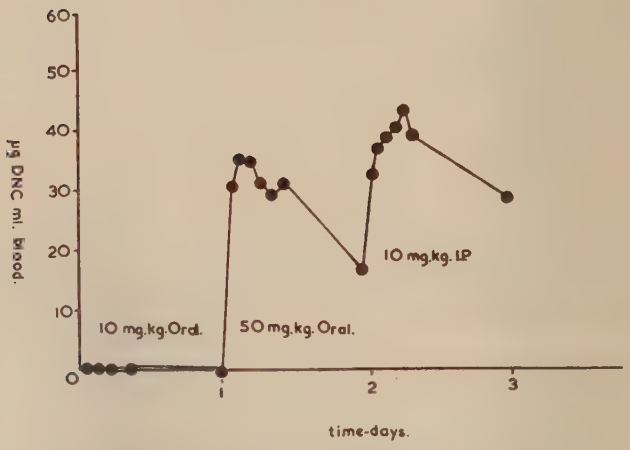


Smith, Smithies and Williams (1953) have identified these compounds as two of the end products of DNC metabolism in the rabbit, and have shown that one of them (1) at least is relatively non-toxic. The appearance of the colour suggests that the amino groups are not acetylated, as in the rabbit, because the acetylamino nitro cresols are yellow and not red. Since 6-amino-4 nitro-o-cresol (1) is readily formed from DNC by ammonium sulphide in vitro, it is likely that this compound may predominate in the rumen content DNC mixtures where sulphides (e.g. H_2S) are known to exist in reasonable concentrations. This interesting aspect is now under further investigation.

Rate of Elimination of Single Doses of DNC in the Ruminant

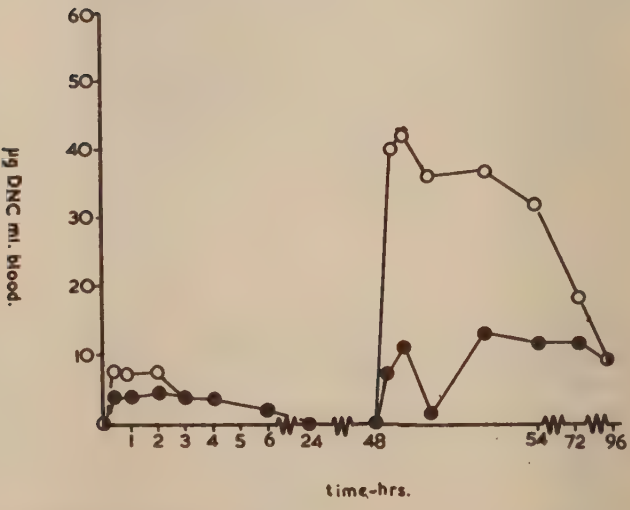
From the results of the previous experiments it was expected that some of the DNC administered orally would be destroyed and

Fig. 2



Blood DNC levels in a goat following three consecutive doses.

Fig. 3

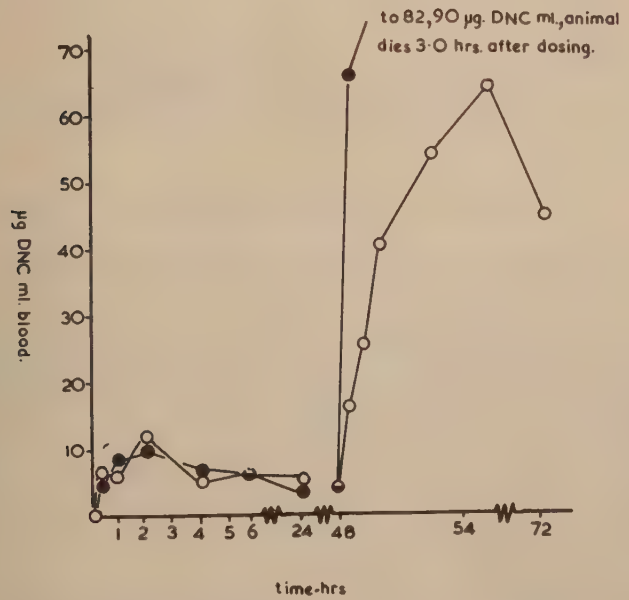


Blood DNC levels in two calves.
o———o 1 mg/kg I.P., followed by 24 hrs. rest, then 10 mg/kg. I.P.
●———● 10 mg/kg Oral, followed by 24 hrs. rest, then 50 mg/kg Oral.

that the resulting blood concentrations would be low. This was borne out by the first group of experiments on the goat, the sheep and the calves. Single oral doses of 10 mg/kg. gave blood DNC values that were insignificant and comparable with single intraperitoneal doses of 1 mg/kg. (Figs. 2, 3 and 4). In the case of the

goat the blood DNC level 24 hours after the second oral dose of 50 mg./kg. was 20 μg./ml. This was further increased to 30 μg./ml. 24 hours later by a third intraperitoneal dose of 10 mg./kg. These results suggest that accumulation of the DNC will occur provided it is absorbed in sufficiently large amounts, and the time intervals between doses are not too long. A further point to note is that the elimination rates of DNC from the blood on the second and third days are very similar. However, they are not rapid enough to result in removal of DNC from the animal in 24 hours. An interesting comparison can be made with the non-ruminants (Fig. 6) where identical doses result in rapid and more complete absorption of DNC. This occurs also in man (Harvey *et al*, 1951). These feeding experiments clearly support the conclusions drawn from the results of the in vitro experiments on the detoxicating power of the rumen contents. Large single oral doses of 50 mg./kg. give rise to higher blood levels of DNC but not as high as would be expected from animals with a simple stomach.

Fig. 4

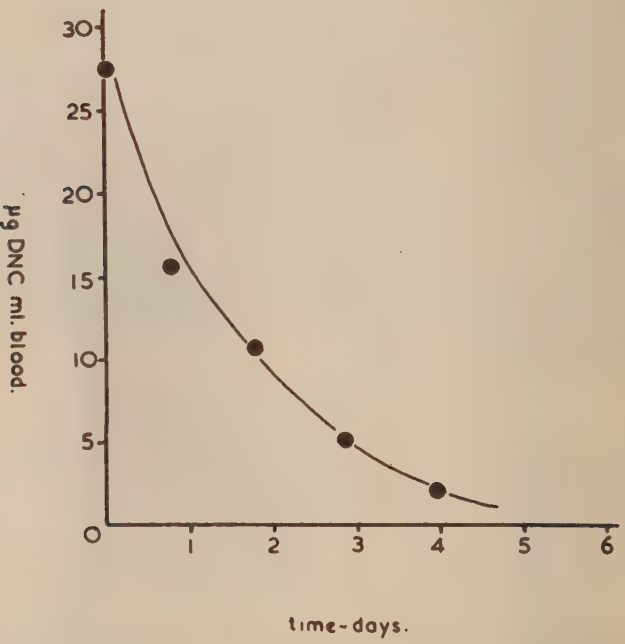


Blood DNC levels in two sheep.
o———o 1 mg/kg I.P., followed by 24 hrs. rest, then 10 mg/kg I.P.
●———● 10 mg/kg Oral, followed by 24 hrs. rest, then 50 mg/kg Oral.

The decay rate of DNC from the blood of the sheep (Fig. 5) is similar to that of the rat or the dog. It is not very rapid, and single large doses may not be eliminated fully until 5 to 6 days have

elapsed. This is borne out by the field experiments summarised in Table 2.

Fig. 5

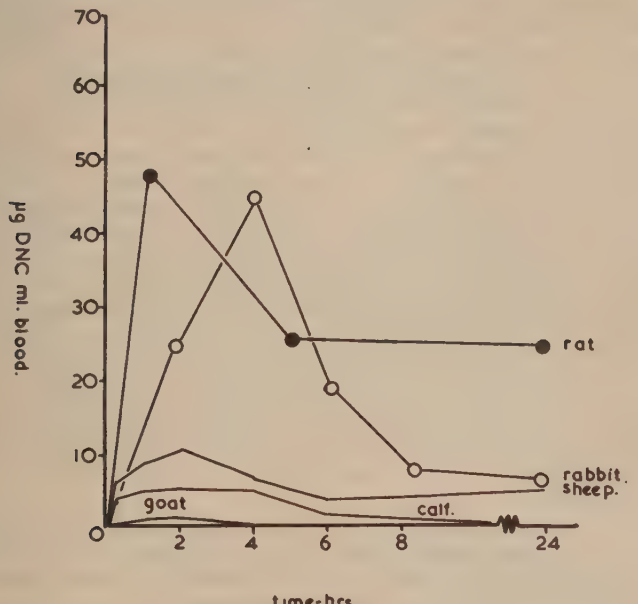


Decay of DNC from the blood of the sheep following a single oral dose of 50 mg/kg.

One somewhat disturbing result was obtained after giving a single 50 mg./kg. dose to a sheep. Shortly after administering the capsules (1 to 2 hrs.) the animal exhibited signs of discomfort, the respiration rate doubled, the blood DNC concentration increased rapidly, and just prior to the death of the animal (3 hrs. after dosing) this was nearly 100 μg/.ml.—a value that is undoubtedly critical for other species. Almost immediately after death a typical DNC rigor set in. Post-mortem examination did not reveal any obvious macroscopic changes, there was no detectable DNC in the trachea or in the lungs and the DNC concentration in the dry rumen contents was low. Clearly the animal had absorbed the DNC rapidly and completely. This suggested that the DNC in the capsules was never in contact with the rumen contents. No explanation can be given to account for this, but one possibility is that absorption might have taken place in the oesophageal groove, whither the entire capsules had found their way before being dissolved and liberating their contents. It is unlikely that this type of poisoning

will arise frequently in natural conditions, as repeated exposures to lower concentrations are a greater possibility.

Fig. 6



Comparison of blood DNC levels in ruminants and small non-ruminants. Data on rat and rabbit taken from King and Harvey (1953 a).

TABLE 2

BLOOD DNC LEVELS IN SHEEP FOLLOWING SINGLE DOSES FIELD CONDITIONS

Animal No.	*Dose mg/kg.*	*DNC µg/ml blood Time after dosing*				
		3·5 hrs.	*1 day*	*2 days*	*3 days*	*4 days*
a 128	1 × 20 Oral	11·0	6·0	5·0	2·0	2·0
a 129	1 × 20 Oral	15·0	8·0	6·15	3·7	2·3
b 130	1 × 20 Oral	5·0	2·5	5·0	1·0	1·0
b 134	1 × 20 Oral	5·0	2·5	5·0	2·5	1·0
136	1 × 10 I.P.	32·0	24·0	20·0	8·0	6·0
137	1 × 10 I.P.	35·0	26·0	20·0	10·0	5·0

Two animals (a) crunched the capsules. This meant that the DNC was spread over the buccal mucosa, upper oesophageal tract, tongue, lips, etc. From these surfaces it may have been absorbed, thus accounting for the initially higher values than those of the other two (b), where the capsules were forced well down into the rumen contents by a pellet gun.

DISCUSSION

The most important fact that emerges from this study is that the rumen contents of bovines are potentially good detoxicating media for DNC. It is possible that the quantitative ability to detoxicate may depend on a large number of factors including the nature of the flora and fauna, the diet of the host and the physical state of the contents. From this it is tempting to assume that such an efficient natural barrier ought to prevent the occurrence of serious DNC poisoning in the ruminant, but it must be remembered that continuous exposures to low concentrations of DNC may disturb essential biochemical processes of the rumen organisms and therefore, indirectly, the health of the host. All this may well occur without the appearance of significant concentrations of DNC in the blood. There are, however, other portals of entry for DNC into the blood stream of an animal. From the limited number of field experiments it is obvious that some absorption takes place through the buccal mucosa, the upper oesophagus, and possibly the lips. An even greater source of danger is the inhalation of a DNC aerosol that will be produced by a down wind drift of a spray. No experiments have been designed to test this pathway of absorption by the ruminant, but it is very unlikely that it will differ markedly from other animals.

From the safety point of view the same broad measures that are employed in safeguarding man should be applied to farm animals. All reasonable precautions should be taken to prevent exposure to DNC, and blood samples should be analysed.

AKNOWLEDGMENTS

I wish to acknowledge with thanks the assistance given by many of my colleagues in the Department of Pathology, especially Mr. J. E. T. Jones, M.R.C.V.S. for carrying out the post-mortem examination of the sheep, and Messrs. P. L. Ingram, B.Sc., M.R.C.V.S., E. F. Hughes and W. J. Gooden for assistance in obtaining blood samples. Also Dr. F. R. Bell of the Department of Physiology for making available many samples of rumenal fluid.

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THE EFFECT OF ADRENALINE, A.C.T.H. AND INSULIN ON THE CIRCULATING EOSINOPHILS OF THE COW

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INTRODUCTION

In a recent review on bovine ketosis, Shaw (1955) found that the subcutaneous injection of 5 ml. 1/1000 adrenaline hydrochloride produced an eosinopoenia in normal cows, whereas in cows with ketosis the same treatment caused no significant change in the number of circulating eosinophils. Shaw attributed the failure of cows with ketosis to show an eosinopoenia to "a temporary exhaustion of the pituitary gland". This evidence has been used to support the treatment of bovine ketosis by the administration of preparations of A.C.T.H. The object of the experiments described here was to repeat and extend these observations.

METHODS

The Ayrshire cows used in these experiments formed part of a herd kept for milk production and were under continuous veterinary supervision. Milk yields were recorded under the normal routine of management. Blood was taken from the external mammary vein into bottles containing a balanced ammonium and potassium oxalate mixture. The circulating eosinophils were counted by Randolph's (1949) method and blood sugar concentration determined by Somogyi's (1952) method used in conjunction with Nelson's (1944) chromogenic reagent.

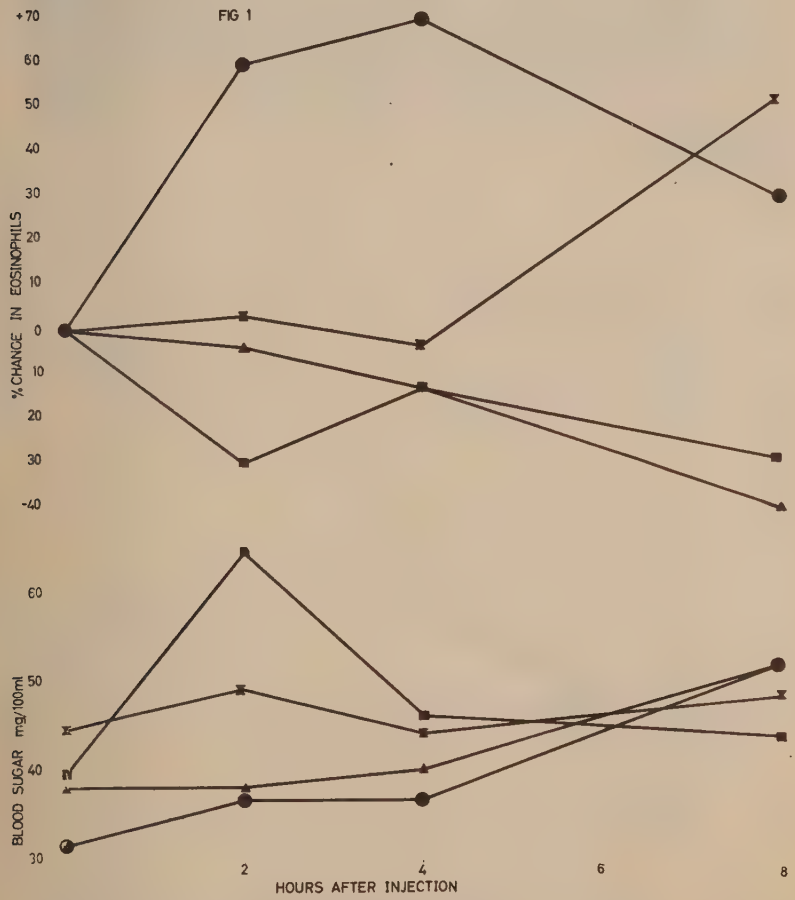
The drugs used were adrenaline hydrochloride 1/1000 (Parke Davis), insulin regular (Allen & Hanburys), the long acting adreno-corticotrophic hormone was cortrophin "Z", (Organon) and the preparation for intravenous injection was corticotrophin (Crookes A.C.T.H.). Where the doses are shown on a body weight basis the approximate weight of an Ayrshire cow was assumed to be 500 kg.

RESULTS

Effect of Adrenaline

Each member of a group of four cows was injected subcutaneously with 5 ml. adrenaline and bled immediately before and at intervals after the injection. The results are shown in Fig. 1. Two of the cows showed an eosinopoenia of less than 50 per cent and two an eosinophilia. However, as the rise in blood sugar was most marked in one of the cows showing an eosinopoenia it seemed possible that too small a dose of adrenaline was the reason for the failure to produce a marked eosinopoenia. However, increasing the dose to 10 ml. adrenaline (25 μg/kg) had no greater effect on either the circulating eosinophils or the blood sugar level of the four cows so

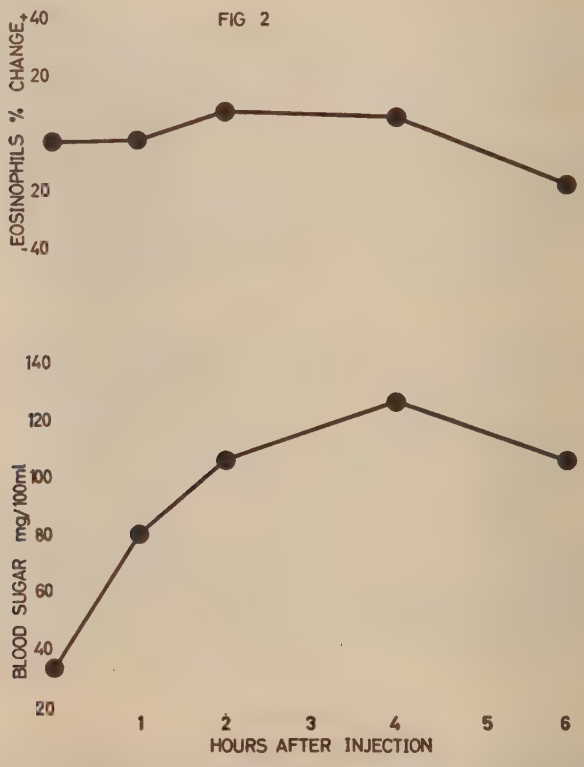
treated. The two cows which failed to show an eosinopoenia had normal appetites and milk yields and their milk was free from ketone bodies.



The effect on blood sugar and eosinophils of four cows of 12·5 μg/kg adrenaline, s.c.

A group of six cows was injected subcutaneously with 20 ml. adrenaline 1/1000 (50 μg/kg). A typical result is shown in Fig. 2. This experiment showed that doses which produced a fourfold increase in blood sugar did not cause any marked eosinopoenia. To eliminate any delay in response due to the slow absorption of adrenaline injected subcutaneously, four cows were injected intravenously with the drug, two receiving 5 ml. (12·5 μg/kg) and two 10 ml. (25 μg/kg). The results are shown in Fig. 3. In each cow the

intravenous injection produced a marked dyspnoea and profuse sweating. Despite the marked pharmacological effects of the drug only one of the four cows showed a significant eosinopoenia (50 per cent change). Moreover, the handling entailed in carrying out repeated venesections produced a 50 per cent fall in eosinophils in one of a group of six cows which did not receive any drug treatment. The milk yield of the cows which received a dose of 20 ml. (50 μg/kg) subcutaneously or 5 ml. (12·5 μg/kg) or more intravenously showed a marked fall.



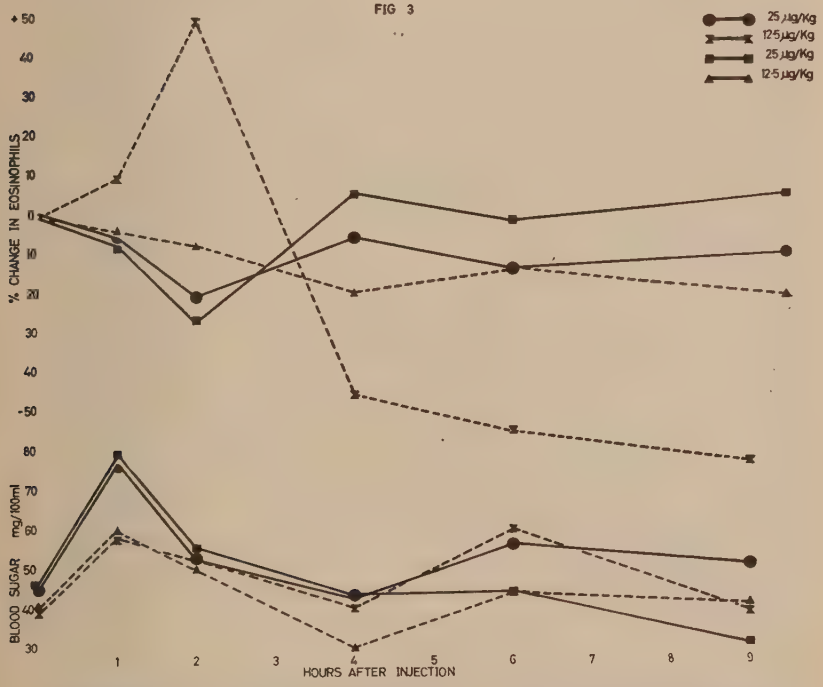
A typical response of the blood sugar and eosinophils to 50 μg/kg adrenaline, s.c.

Effect of A.C.T.H.

With the failure of adrenaline to produce consistently an eosinopoenia, it seemed desirable to show that the eosinophils of the cow would respond to injections of A.C.T.H. Ten cows received A.C.T.H. in two forms and by two routes of administration. The results are given in Table 1. They show that A.C.T.H., in adequate dosage, produced a fall in circulating eosinophils of more than

50 per cent. Intravenous injections, which eliminated any delay of the response due to absorption of the preparation, produced the effect in 2 to 4 hours. A.C.T.H. in doses which produced an eosinopoenia caused a rise in the blood sugar of about 10 per cent to 20 per cent of the initial value. The doses of A.C.T.H. used in these experiments had no effect on the milk yield.

FIG 3



The effect on the blood sugar and eosinophils of four cows of adrenaline injected intravenously.

Effect of Insulin

The effect of hypoglycaemic "stress" was studied on a group of five cows. The results are shown in Table 2. Insulin in a dose of 1·25 units/kg injected intravenously was sufficient to produce hypoglycaemia. The mean initial value of the blood sugar of the five cows was 41·5 ± 2·9 (S.E.) and one hour later 14·8 ± 2·3 (S.E.) mg. glucose/100 ml. blood. This marked hypoglycaemia did not appear to cause the animals any distress. The effect on the eosinophils was very variable although some degree of eosinopenia was produced. The dose of insulin used had no effect on the milk yield.

TABLE 1

PERCENTAGE CHANGE IN EOSINOPHILS AFTER THE INJECTION OF A.C.T.H.

Cow	*Dose*	*Route*						
			Long acting preparation					
1	20 i.u.	i/m	—14	—10	—10	+7	—40	
2	,,	,,	—20	+ 2	—57	—35	—37	
3	,,	,,	— 3	— 9	—16	—33	—77	
4	40 i.u.	,,	—27	—88	—92	—93	—73	
5	,,	,,	+10	—57	—78	—84	—78	
6	,,	,,	—24	—63	—74	—88	—82	
Hours after injection			3	6	9	12	24	
			Short acting preparation					
7	25 i.u.	i/v	+23	— 5	+ 9	— 7	—12	—32
8	,,	,,	+20	—14	—31	—68	—49	—43
9	50 i.u.	,,	—12	—71	—51	—44	—20	—28
10	,,	,,	+ 5	—24	—57	—74	—62	— 9
Hours after injection			1	2	4	6	8	10

TABLE 2

THE EFFECT OF INSULIN ON CIRCULATING EOSINOPHILS

Cow No.	*Percentage change*					
1	—21	—18	—27	—11	—47	—47
2	— 3	+ 5	—26	—28	—26	—31
3	—15	+11	—38	—10	—50	—31
4	—31	—15	—35	—22	—12	0
5	—18	—44	—41	—29	— 9	—35
Hours after Injection	1	2	4	6	8	10

DISCUSSION

The results of these experiments clearly showed that cows with normal appetites and milk yields and whose milk was free from ketone bodies failed to show an eosinopoenia following the subcutaneous injection of 5 mg. adrenaline. As such cows could not be regarded as suffering from ketosis, the results were contrary to those of Shaw, Hatziolos, Leffel, Gill and Chung (1952) and Shaw (1955). Even when the dose of adrenaline was greatly increased an eosinopoenia was produced only occasionally. The cow seemed to resemble the sheep in the response of its circulating eosinophils to adrenaline as the dose of the drug necessary to produce an eosinopoenia in sheep was big enough to cause a five-fold increase in blood sugar (Zarrow, Denison, Rosenburg, Mann and Neher, 1952). The eosinophils of the horse (Alexander and Ash, 1955) behaved in a similar fashion as they were unaffected by adrenaline in doses up to 25 μg/kg, whereas rats responded to doses of adrenaline too small to cause a rise in blood sugar (Gershberg, Fry, Brobeck and Long, 1950). Moreover, a dose of 0·4 mg. adrenaline has been recommended as adequate to produce a 50 per cent fall in circulating eosinophils in man (Recant, Hume, Forsham and Thom, 1950). The absence of response to adrenaline shown by the circulating eosinophils of the cow was not due to their inability to respond to A.C.T.H. as injections of adequate doses of this hormone caused a fall in the eosinophils of more than 50 per cent of their initial value.

There appeared to be an interesting difference between the sheep, horse and cow in the response of the circulating eosinophils to hypoglycaemia. Sheep (Zarrow *et al.*, 1952) and horses (Alexander and Ash, 1955) showed an eosinopoenia of 90 per cent and about 75 per cent respectively in response to insulin hypoglycaemia, whereas the cows in the experiments described here showed a barely significant response (50 per cent change). This difference was difficult to explain, as sheep, cows and horses all failed to show clinical signs of hypoglycaemia, and it might be expected that their circulating eosinophils would behave similarly in response to hypoglycaemia. This difference between sheep and cows was particularly interesting since both are ruminants and depend largely on the lower volatile fatty acids as the main source of carbohydrate.

The milk yields of the cows under experiment were only affected by large doses of adrenaline, which caused them to fall. Insulin and A.C.T.H. had no effect.

CONCLUSIONS

Adrenaline in doses of 25 μg/kg injected subcutaneously failed to produce a significant eosinopenia. In one cow in a group of six, an eosinopoenia was produced by 50 μg/kg.

A.C.T.H. in adequate dosage produced an eosinopoenia within two to four hours.

Insulin (1·25 units/kg) caused a marked hypoglycaemia, no clinical signs and an inconsistent effect on the eosinophils.

The high doses of adrenaline caused a fall in milk yield, whereas insulin and A.C.T.H. had no effect.

The results do not support the thesis that only cows with ketosis fail to show an eosinopoenia after the administration of adrenaline and are suffering from a "temporary exhaustion of the pituitary gland".

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A. lumbricoides *ova*. These were obtained from the terminal part of the uterus of fresh adult female worms. Eggs were isolated from the uterine tissue by means of a high speed emulsifier ("Atomix") and the resulting suspension was incubated in one per cent formol saline at 27°C for 40 days. Eggs were administered orally by pipette.

Ascaris larvae. These were obtained from the lungs of rabbits 8 days after they had received a large dose of infective ova. The lungs were minced and the larvae recovered in a Baermann apparatus. Tissue debris was removed by washing larvae in a copper sieve (aperture=37μ). Extracts of larvae were made by disintegrating them in a small quantity of saline in a large Griffiths tube. The metabolic products were obtained by incubating the larvae, obtained from rabbit lungs eight days after infection, in Stoll Ringer saline at 37°C for 24 hours. Three 24 hour samples of metabolic products could be obtained from an individual batch of larvae without danger of marked contamination by larval tissue antigens due to disintegration of larvae.

Antigens. Antigens for the complement fixation test were obtained from tissues dissected from fresh adult worms. The tissues were "freeze-dried" and pulverised in a ball-mill. Antigen solutions were arbitrarily standardised to contain 0·25 milligrammes of protein per ml.

Sera. All sera were inactivated at 56°C for 30 minutes. They were stored at — 20°C until a complete series was available and the serum samples from individual rabbits were titrated simultaneously. Guinea pig complement was preserved by the method of Richardson (1941).

Serological tests. The haemolytic test for the detection of heterophile antibody was based on the 50 per cent end point technique with colorimetric estimation of haemolysis of a standardised suspension of sheep red cells equivalent to 0·5 ml. of a 2·5 per cent suspension in the presence of four 50 per cent units of complement. The activity of any serum was recorded in 50 per cent units which were read from plots of $\log \frac{y}{1-y}$ against log x. The linear relationship of these values is given by the equation $x = K\left(\frac{y}{1-y}\right)^{1/n}$ (von Krogh, 1916), where x is the amount of antiserum, y is the degree of haemolysis expressed as a fraction of 1, and K and 1/n are constants. A detailed account of the use of this method is given by Taliaferro and Taliaferro (1950).

Complement fixation tests using various antigens were carried out on the 50 per cent end point basis and were based on methods described by Wadsworth, Maltaner and Maltaner (1931, 1935).

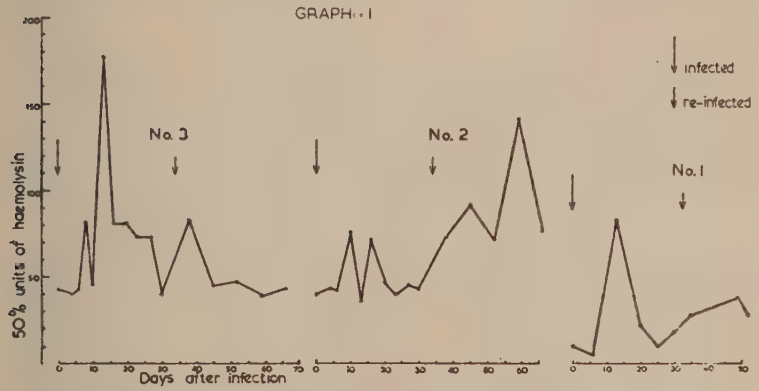
Agglutination of sheep erythrocytes was tested for by exposing a 0·4 per cent suspension of cells to an equal volume of serially diluted rabbit serum at 37°C for 30 minutes. The cells were deposited by centrifuging and agglutination was determined by microscopic examination of a sample of the deposited cells.

RESULTS

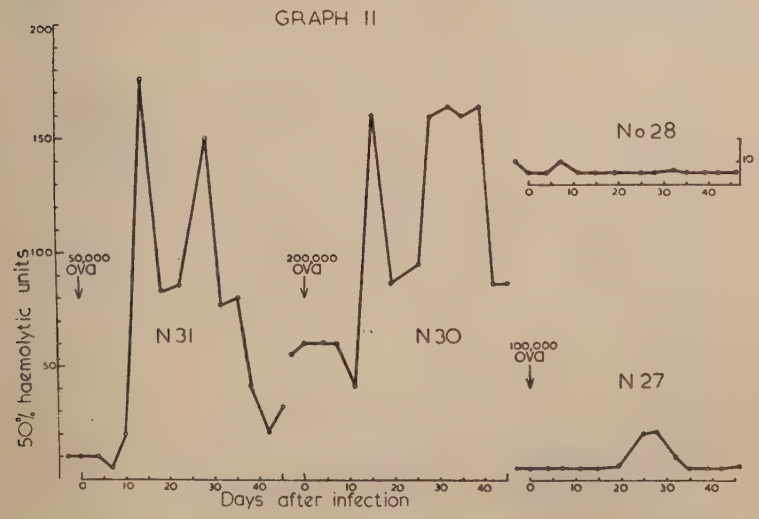
Rise in Heterophile Titre in Rabbits Infected with A. lumbricoides

Three rabbits (N1, N2, N3) were given 100,000 ova per os and the pre- and post-infection titres of haemolytic antibody to sheep

cells were determined in serum samples taken at intervals of 7 days. Reinfection with 100,000 ova was carried out 32 days later. The responses are demonstrated in Graph I. To confirm the results in the first three rabbits a further 3 rabbits were infected with 50,000, 100,000 and 200,000 ova per os respectively and a fourth rabbit was used as a control. Serum samples were obtained before and after infection at intervals of 3 or 4 days. The levels of heterophile antibody are presented in Graph II.



Graph I. Forssman antibody levels in three rabbits infected with 100,000 *A. lumbricoides* ova and reinfected with 100,000 ova 32 days later.

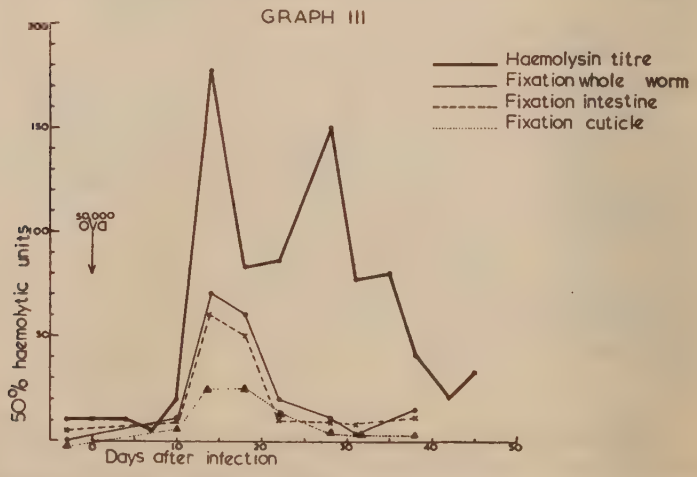


Graph II. Forssman Antibody levels in three rabbits infected with *A. lumbricoides* ova and in one control rabbit (No. 28).

From these groups it can be seen that the normal (pre-infection) heterophile antibody titres in some rabbits (2, 3 and N30) are substantial. This normal antibody has been commented upon by Taliaferro and Taliaferro (1950) who mentioned the tendency at times for such antibody to lack the ability to produce 100 per cent haemolysis. This has not been noted in the present work.

With the exception of one animal (N27), an initial peak of heterophile titre occurred 10 to 15 days after infection though an accurate determination of the time of initial peak titre would necessitate more closely spaced sampling. The titre of the control rabbit was remarkably constant and is in accord with the findings of Mackie (1925) regarding heterophile levels in normal rabbits.

The results in Graph I would suggest that reinfection quickly stimulated an anamnestic type of response. However, there are reasons why the secondary response in *Ascaris* infection would be expected to differ from that usually associated with antibody production, due to immune mechanisms preventing the migration and development of larvae. Graph II indicates that a secondary response does not necessarily depend on reinfection. The characteristic haemolytic heterophile antibody response consists of an initial marked rise to peak titre to be followed by a decay of antibody fitting a discontinuous curve (Taliaferro and Taliaferro 1950). The occurrence of a secondary response 28 days after infection may indicate restimulation from Ascaris larvae which have been trapped in the tissues and are undergoing disintegration. The delayed response in rabbit N27 (Graph II) could be explained on this basis. A situation comparable to this has been noted in the antibody levels in schistosomiasis (Fairley and Jasudason, 1930).



Graph III. Comparison of Forssman antibody levels with complement fixing antibody levels (using different antigens) in a rabbit infected with 50,000 *A. lumbricoides* ova.

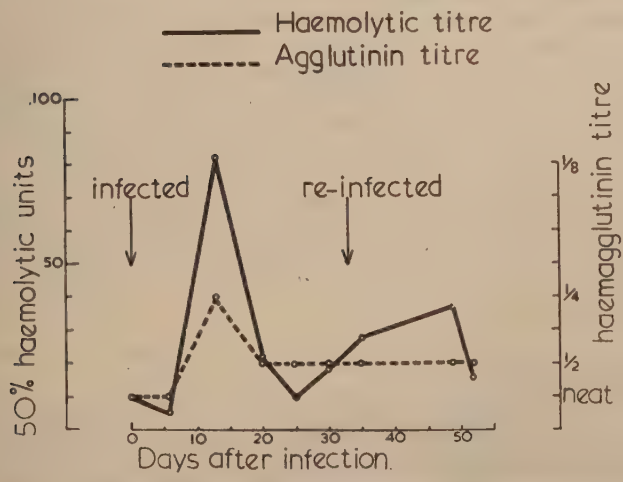
To see whether the heterophile antibody titre followed other true anti-Ascaris antibody titres, complement-fixation tests were carried out on the sera of rabbit N31 using several antigens. The results are presented in Graph III where it is seen that complement-fixation titres generally followed the variations of the heterophile antibody. This suggested that the heterophile antibody could be used as a suitable "marker" antibody in the study of antibody production in *A. lumbricoides* infection. Subsequent work has been planned with this in mind.

Identification of the Heterophile Antibody

It appeared that this heterophile antibody possessed the properties of the classical antibody of Forssman (1911) which has as some of its characteristics, in addition to the haemolysis of sheep cells the ability to be absorbed by guinea pig kidney emulsion, a low ability to agglutinate sheep red cells and an absence of haemolysis for bovine red cells.

The heterophile antibody produced in Ascaris infection of rabbits could be absorbed completely with guinea pig kidney emulsion and also with fowl erythrocytes. Verification as to the identification of the antibody as typical Forssman was given by the absence of haemolysis for bovine erythrocytes and a very low haemagglutination for sheep cells. A typical erythrocyte agglutination titre compared with the haemolytic titre is given in Graph IV.

GRAPH IV



Graph IV. Comparison of Forssman antibody levels (haemolytic titre) with haemagglutin action levels in rabbit infected with *A. lumbricoides* ova.

Site of the Forssman Antigen within A. lumbricoides

Using known Forssman sera (raised against guinea pig kidney and fowl red blood cells) various body tissues of the adult worm and living and dead larvae were used for absorption tests. The results are summarised in Table 1. The complete absorption of the antibody by Ascaris larvae from guinea pigs is probably explained by the fact that with such preparations it is impossible to free larvae completely from host tissues and the presence of guinea pig lung tissue would result in removal of Forssman antibody.

Using larvae from rabbits, and thus avoiding contamination by native Forssman tissue, the most efficient absorbing agent for anti-guinea pig kidney serum was an extract of disintegrated larvae. Living or dead larvae or the metabolic products of living larvae were relatively inefficient for absorption, but did show some evidence of Forssman antigen. With the anti-fowl-erythrocyte serum, living larvae proved the most efficient absorbing agents, an extract of larvae proving less efficient. This may indicate the existence of two antigen types in Ascaris larvae, one with a major specificity for guinea pig kidney and the other with a major specificity for fowl erythrocytes. Irrespective of this, however, it is clear that the Forssman antigen is present within the larvae and is also produced by the living larvae. The relative inability of metabolic products to absorb Forssman antibody may be simply quantitative in effect. However, some qualitative differences may be expected since recovery of these metabolic products necessitates incubation of large numbers of larvae at 37°C for 24 hours and destruction of antigens by enzymes may occur during the process. Absorption with adult worm material indicates that the intestine is the main source of Forssman antigen and it may be that a similar site for the antigen occurs in the larvae. Disintegration of larvae would then be necessary for the liberation of large amounts of Forssman antigen.

High Forssman antibody levels can be induced in rabbits by the injection of adult Ascaris intestine or a larval extract. This is in distinction to the Forssman antibody response induced by whole worm preparations which cause only a slight elevation of titre. Such whole worm preparations are unlikely to contain much Forssman antigen since the intestine represents but a fraction of the total bulk of the worm and this would explain the failure of Grana (1945) to produce heterophile antibodies in rabbits injected with such materials.

Stage of A. lumbricoides *Infection Associated with the Stimulation of Forssman Antibody*

It was of interest to know at which stage during the migration of larvae in experimental animals there was the most marked stimulation of the Forssman antibody. This in turn might give some indication as to the stages which are responsible for the production of antibodies associated with protection against infection. The use

TABLE 1

ABSORPTION OF HETEROPHILE ANTIBODIES BY ASCARIS TISSUES

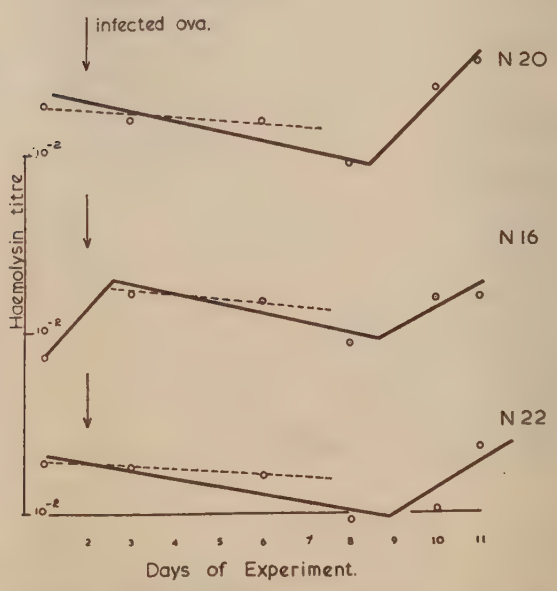
Absorbant	*Antisera*							
	Anti-guinea pig kidney		*Anti-guinea pig kidney*		*Anti-fowl rbc*		*Anti-fowl rbc*	
	S	*A*	*S*	*A*	*S*	*A*	*S*	*A*
Extract whole adult Ascaris					3033	1280		
Extract Ascaris cuticle	1280*	640*	876	730	—	—	—	—
Extract Ascaris testes	1280*	640*	876	730	—	—	—	—
Extract Ascaris intestine	1280*	40*	876	NEG	—	—	—	—
Extract Ascaris ovaries	1280*	640*	876	665	—	—	—	—
Larvae ex-guinea pig lungs	1280*	NEG*	—	—	—	—	5120*	NEG*
Larvae ex-rabbit lungs (1 hour at 37°C)	1008	920	869	678	3033	2496	5210	2150
Larvae ex-rabbit lungs (1 hour at 37° C+18 hours at 4°C)			876	665	3033	1866	4966	2662
Larvae ex-rabbit lungs (1 hour at 37°C)			769	499	—	—	—	—
Larvae ex-rabbit lungs (1 hour at 37° C+18 hours at 4°C)			704	524	—	—	—	—
Killed larvae ex-rabbit lungs (1 hour at 37°C)			768	533	—	—	—	—
Killed larvae ex-rabbit lungs (19 hour at 37°C+18 hours 4°C)			704	462	—	—	—	—
Extract disintegrated Ascaris larvae ex-rabbit lungs			590	18	2739	1369	3046	2176
Extract disintegrated larvae ex-rabbit lungs			640	21	—	—	—	—
Metabolic products larvae ex-rabbit (1 hour 37°C)			590	287	3033	2918	2739	2176
Metabolic products larvae ex-rabbit (1 hour 37°C+18 hours 4°C)			640	355	3046	2662		

S=Absorbed with saline. A=Absorbed with antigen.
The figures refer to the activity of sera expressed in 50% haemolytic units.
All absorptions were done with equal volumes of reagents at room temperature for 1 hour except where otherwise stated.
* Visual estimation of 50% haemolysis.

of the haemolytic Forssman antibody and in particular the secondary response of it, provided a useful method of assay since a substantial body of information is available about it (Taliaferro, 1955).

Moderate antibody responses to guinea pig kidney, the sera usually containing less than 1000 units of activity, were induced in rabbits. Serum samples were obtained frequently and when the characteristic straight line decay of antibody preceding the plateau was established (Taliaferro, 1955) some of the rabbits were given large doses of Ascaris ova per os. Preliminary tests in three rabbits (N16, N20 and N22) given 200,000 ova (Graph V) showed that a secondary response could be expected within 8 days after infected and in subsequent experiments serum samples were obtained daily for the first 4 days and twice daily for the next three days and daily thereafter. The haemolysin decline in the three rabbits in Graph V did not follow the expected course, the reading on the 8th day of the experiment being exceptionally low. The probable course of the haemolysin decline is indicated by the discontinuous line and it is possible that the figures for the 8th day represent an in vivo neutralization of antibody by antigen produced by the migrating larvae.

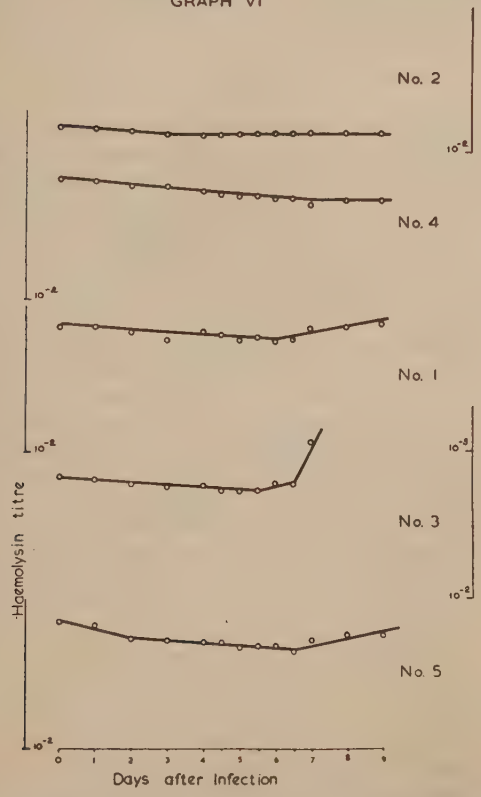
GRAPH V



Graph V. Secondary response in Forssman antibody levels in three rabbits infected with 200,000 *A. lumbricoides* ova on day 2 of experiment. The discontinuous lines represents the anticipated course of antibody decay in the absence of infection with ova.

To overcome any interference with the normal haemolysin decline a further three rabbits were given 50,000 ova per os, whilst 2 rabbits remained as controls. The non-infected rabbits (N2 and N4, Graph VI) maintained the straight lines of decay which are characteristic of the high molecular Forssman antibody. The three infected rabbits (1, 3 and 5, Graph VI) showed a secondary response between the sixth and the seventh day after infection. The induction period in the secondary response of Forssman antibody production is short and is roughly two-thirds of the initial induction period (Taliaferro and Taliaferro 1952). An induction period of two days could not unreasonably be said to be applicable in the present work. Using this figure it would indicate that the primary stimulation of the secondary response occurred between days 4 and 5 after infection. It is at this time during infection that Ascaris larvae commence a spectacular period of growth and this is also associated

GRAPH VI



Graph VI. Secondary response in Forssman antibody levels in two control rabbits (2 and 4) and in 3 rabbits (1, 3, and 5) given 50,000 *A. lumbricoides* ova per os.

with increased migratory behaviour (Soulsby, 1956). In addition, the moulting of larvae from second stage to third stage is initiated during this period with the result that almost all larvae are at the third stage on the sixth day (Roberts, 1934). Unfortunately, this technique will not indicate the quantitative antigen production beyond this period, but the present results indicate that the period of, and immediately prior to, the second ecdysis is the earliest time at which to expect any marked amount of antigen to be produced and may well be an important period for the release of antigens which eventually will stimulate "protective" antibodies.

Role of Forssman Antibody in Immunity

During this series of experiments rabbits with high titres of antibodies against fowl red blood cells and guinea pig kidney were infected with Ascaris ova. None of the rabbits showed any evidence that high heterophile antibody titres influenced the Ascaris infection. When Ascaris larvae were placed in such sera in vitro they suffered no apparent ill effects and were viable after overnight incubation.

DISCUSSION

Since rabbits with high anti-Forssman titres were not protected against Ascaris infection it is considered that the antigen is not concerned in the stimulation of "functional" antibodies. Nevertheless, the antibody can serve as a useful tool in delineating the various aspects of the antibody response since it can be easily detected and determined with accuracy by the 50 per cent haemolysis end point. As such it serves as a "marker" antibody.

Its main situation is in the intestinal tissue of the adult worm and somewhere within the body of the larvae. It is also present, however, in small amounts in "metabolic products" obtainable from living larvae. Any marked stimulation of the antibody would therefore be expected to be associated with disintegration of larvae or some period of marked metabolic activity. The calculated time of the stimulation of the secondary response coincides with the latter expectation, i.e. during second ecdysis. As it is unlikely that all larvae undergo the second ecdysis with impunity, disintegration of larvae could contribute to the stimulation of the Forssman antibody. It is not possible to say that the period between day 4 and 5 after infection is the main period of antigen release and it will be necessary to follow such antigen release by other methods. It is also important to know whether other antigens, more "functional" in nature, are released at the same time as the Forssman antigen.

Antibodies detectable by complement-fixation follow the same trend as the Forssman antibody (Graph III) and it would appear that a general release of antigens occurs along with that of the Forssman.

The antibody induced by Ascaris infection appears to be closely related to the classical Forssman type. The absence of marked agglutination of sheep cells distinguish it from other heterophile

types, especially the "Paul Bunnel" type. The presence of a high haemolytic Forssman titre in man, in the absence of serum therapy, may well merit consideration from the point of view of Ascaris infection.

CONCLUSIONS

Forssman antibody increased in titre during *A. lumbricoides* infection in the rabbit. The homologous antigen is present in the tissues, chiefly the intestine of the adult worm, and in an extract of larvae. It is also present in the excretions and secretions obtained from the larvae. Using the secondary response to an established Forssman antibody system in vivo it was possible to indicate that the period of the second ecdysis of Ascaris larvae is the earliest period at which to expect a marked release of antigens.

The Forssman antibody was found to have no protective action against infection.

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MORTALITY OF NEW-BORN PIGS ASSOCIATED WITH A MATERNAL DEFICIENCY OF VITAMIN A

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INTRODUCTION

Although it is over twenty years since Hale (1933, 1935) described the birth of blind and deformed piglets from gilts fed a diet deficient in vitamin A, there is still debate in Britain on the extent to which avitaminosis A might be concerned in the neonatal pathology of the pig (Harding and Done, 1956; Watt and Barlow, 1956b). During 1956 we were asked to examine four herds in which piglets were dying shortly after birth or, although viable, were suffering from microphthalmia. In all these herds a diagnosis of avitaminosis A was reached and the object of this paper is to describe the clinical and pathological features of the field condition. In particular, we refer to one herd, the first examined, where the disease as first presented suggested an infective aetiology. The four herds are referred to as herds A, B, C and D, and their respective histories are given in an appendix. The main text contains an integrated account of the condition based on all the material examined.

MATERIALS AND METHODS

Estimation of vitamin A. For liver analyses the method of Davies (1933) was used and for serum levels a modification of the method of Kimble (1939).

Post-mortem examinations. All post-mortem examinations were made within 36 hours of death, but in many instances the interval was less than 18 hours. Several piglets were killed by cutting the throat vessels in the laboratory and examined at once. Tissues for histological examination were fixed in 10 per cent formol saline or Zenker's fluid; paraffin, frozen or celloidin sections were then prepared. The stains used were haematoxylin and eosin, van Gieson, Sudan III, Lendrum's phloxine tartrazine, Giemsa, Mann's eosin methyl blue and Marchi. The tissues examined were as follows: pituitary gland, parotid gland and duct, submaxillary gland, adrenal, colon, small intestine, stomach, eye, thyroid gland, spinal cord, lung, liver, heart, testis, cervix, ovary, skin including mammary gland, pancreas, kidney, spleen, rib junction and jaw bone with developing teeth.

GENERAL OBSERVATIONS

Aetiology

The conclusion that a deficiency of vitamin A was the primary cause of the abnormalities described derives from evidence of three main types—circumstantial, analytical and experimental. The circumstantial evidence is based on the diets fed during pregnancy

to the affected sows and in herds A, B and C these were grossly deficient in vitamin A; furthermore, in these same three herds the animals that were fed vitamin A, either concurrently or at a later date, gave birth to normal litters. The effect of improving the diet was particularly instructive in herd A; for whereas many litters had been born dead before vitamin A was incorporated, a succession of blind, but otherwise healthy piglets was born during the period immediately following the dietary change, and it was not until later that completely normal piglets were produced.

The analytical evidence depends mainly on the figures for the concentration of vitamin A in the livers of the piglets at birth. In herd A only one litter was so examined and this, unfortunately, was not until the piglets were being born viable but blind. However, the values of between 4 and 5 i.u. per g. in this instance support the general conclusion. In herd B, three piglets were examined from two litters and seven piglets from a third. All these animals had died shortly after birth and in none was there any appreciable concentration of vitamin A in the liver.

The experimental results, obtained with the four affected sows of herd B, were unequivocal. The animals were moved to Cambridge and re-mated to our boars after two of them had received over a short period vitamin A palmitate in arachis oil. All four sows were fed the same diet that had produced non-viable or blind piglets on the home farm and the treated pair continued to receive vitamin A throughout gestation. The litters born from the treated sows were vigorous and apparently normal, whereas no piglet survived from the litters of the deficient sows. One piglet was sacrificed from each of the normal litters and both livers were well-stocked with vitamin A. By comparison, the piglets out of the untreated sows had no vitamin A in their liver (Table I, appendix).

Clinical Signs

The clinical signs can be conveniently classified into three groups that accord with the severity of the condition. In the most severe form the piglets were born at full-term but were either still-born or moribund. In such litters some piglets were under-developed, but an insufficient number of litters has been seen to say whether this lack of development might be more common than in the piglet population generally. Some of the still-born piglets showed severe ascites. The piglets that lived for only a few hours were listless and very weak; they had little interest in suckling and commonly crawled well away from the sow. There was a marked tendency on the part of slightly stronger piglets to burrow as deeply as possible into the bedding. None had the strength to walk normally and many lay on their sides around the hindquarters of the sow until they died. In the latter instance it was usual for the piglet to make slow paddling movements with the legs while squawking intermittently. Such plaintive squawking by many pigs in an

affected litter is particularly characteristic. Another very common feature in those piglets that died shortly after birth was a lack of tone and colour in the skin, so that flakes of membrane continued to adhere to the body (Fig. 1). Sometimes the presence of subcutaneous oedema gave the animals a rounded appearance. In the litters where the disease was naturally-occurring, and where the necessary examination was made, all the moribund piglets had eye defects, usually with either marked entropion (Fig. 2) or exophthalmos. The lids were often gummed together with a brown wax-like material that also caused the eyelashes to be stuck together in groups; this material tended to accumulate at the inner canthus of the eye (Fig. 3). The contents of the orbit were commonly sunken owing to the existence of microphthalmia and vestigial eyeballs (Fig. 4). Corneal opacity was seen (Fig. 5) and in one animal the lids were firmly fused to the cornea. In the two affected litters that were produced experimentally, however, there were no eye abnormalities of any type; this fact is of special interest and is discussed later. Our cases do not permit any discussion of the more severe forms of the condition where the litter might be aborted or resorbed.

When the condition was less severe the pigs were more vigorous at birth and they appeared normal except for gross eye defects similar to those seen in the still-born pigs. In the third and mildest form of the disease the only obvious defects were again in the eye, but there was now no involvement of the lids and the eyeball was of normal shape and size; the defects were mainly in the position and extent of the iris so that the sclera was either too extensive (Fig. 3) or unusually restricted. Piglets that were vigorous at birth were usually reared, but there were some cases in herd A where the deficiency appeared to worsen by about 3 weeks of age. These animals developed nervous signs and posterior paralysis, and they were probably similar to the cases produced by Nordfeldt (1945).

It is noteworthy that of the sows examined none that farrowed affected piglets showed any obvious clinical abnormalities, not even the two sows of herd B that lost two successive litters each. Some of the gilts of herd C became weak in the hindquarters early in gestation and such signs could have been referable to avitaminosis A, but these particular animals were slaughtered before the herd was first examined.

Gross Pathology

Twenty-seven piglets were examined from herds A and B, and of these ten were still-born. On opening the carcases of both still-born and live animals the subcutaneous tissues, particularly the axillary and submaxillary regions, were seen to be infiltrated with a yellow, gelatinous fluid. The musculature was moist and an unusual, very light-brown colour. The body cavities, and especially the pericardial sac, contained large quantities of a sero-fibrinous straw-coloured fluid, which also permeated and thickened the

mesentery. Occasionally the volume of fluid in the cavities was sufficient to produce obvious distension. The hearts appeared larger than normal and had a pale, slightly-boiled appearance. The epicardium showed petechial haemorrhages, most marked on the fat and in the coronary grooves. The gall bladders were distended with a dark, viscous bile and the walls were oedematous. Fibrin was often present on the peritoneal surface of the liver, usually in a network but sometimes as a solid sheet. In piglets that had not suckled, the stomach contained a glairy mucus; this was sometimes abundant and then it was more fluid. The spleens were enlarged and turgid from venous congestion. The mesentery of the colon often contained a gelatinous, semi-solid material similar to that seen in cases of so-called bowel oedema of older pigs, and there was also oedema beneath the peritoneal coat of the intestines. On longitudinal section both the medulla and cortex of the kidneys frequently contained cysts that varied greatly in size. In a few piglets there was no obvious renal medulla and a somewhat gelatinous tissue was present instead. Numerous subcapsular haemorrhages were present but these are normally seen in the kidney of the new-born pig. The kidneys were absent in one still-born pig and vestigial in two live-born animals. There were no gross changes in the central nervous system, the skin or the skeletal system.

In the still-born piglets the lungs, which were a deep-bluish colour, were collapsed and small, and in three cases there was marked hypoplasia of the left lung, which was completely collapsed and only about a third of the normal size. One piglet had a diaphragmatic hernia, a portion of the liver being in the thorax. Striking changes were present in the livers, which were yellowish-grey in colour and mottled by large darker areas and small subcapsular haemorrhages.

In contrast to the still-born animals, the piglets that breathed did not show striking liver changes, but the liver was usually very congested and oozed blood when cut. The chests of the live-born animals also contained fluid, but only parts of the lungs were collapsed. In many cases there was obvious pulmonary oedema and sometimes congestion with petechiae. The small bowel in two live-born pigs showed widespread venous congestion and was a deep plum-colour.

The experimental cases were generally similar to those received from the field, but the extensive oedema was now much more a feature of the disease. In the still-born animals the livers, which were mottled as before, contained haemorrhages and these were most obvious at the edges of the lobes.

Histopathology

Lungs. A number of different changes were seen in the lungs, often within a single litter. In the still-born animals the lungs were collapsed,

the alveolar walls being in close apposition. Usually there was evidence of venous congestion and pulmonary oedema, which was sometimes striking (Fig. 6). The alveolar spaces and smaller bronchioles were distended with an eosinophilic material that was granular in appearance. In all the piglets the bronchial epithelium showed some degree of change. There was invariably vacuolation of the cells, together with a proliferation of epithelial layers which produced heaped up areas of cells (Fig. 7). The epithelial cells were irregular in shape and in some the nuclei had disappeared or seemed to be undergoing change. In no case, however, was there a distinct metaplasia to a squamous epithelium. In some animals the bronchial epithelium had desquamated and the bronchial lumen contained debris; these changes were associated with a foetal pneumonia (Fig. 8), with plugs of cells in the alveoli and bronchioles, and thickening of the alveolar walls. Mononuclear cells were prominent in these sections but polymorphonuclear leucocytes were not common.

Liver. The histology of the liver was quite different in still-born and live-born piglets. In the former, the changes were those of decomposition, so that in many areas the liver architecture had been lost and there remained only an indistinct stroma filled with red cells. In less advanced cases there was extensive fatty change and marked venous congestion. In the animals that breathed, however, venous congestion was a fairly constant finding, the sinusoids being prominent and engorged with red cells. In many instances there was an increase, sometimes very marked, in the numbers of round cells around the small bile ducts (Fig. 9). The liver cells contained glycogen vacuoles resembling fat spaces but although fat droplets could be demonstrated in frozen sections stained with Sudan III, fatty changes were not marked. It had been observed previously that the liver of the new-born pig does not show lobulation and this finding was confirmed in both the affected and the control animals.

Myocardium. The only consistent change was some slight degree of separation of the muscle bundles by oedema fluid.

Pancreas and salivary glands. Generally there was some lymphocytic infiltration around the ducts of all these glands; it varied in degree, but was always more pronounced than in the two control piglets. The duct epithelium was vacuolated and in places several layers deep, but no metaplasia was noted (Fig. 10). The pancreas seemed slightly deficient in stroma, and the acini were separated by clear spaces. These probably represented oedema spaces. The islets of Langerhans were not easy to find, but they were no more obvious in the control piglets.

Kidneys. Developmental abnormalities were common. The vestigial kidneys were small and probably non-functional. The cortex was fairly well developed, with many glomeruli, but the medulla was not well-defined and the renal tubules were scanty. This medullary hypoplasia was best seen in kidneys where the medulla consisted of two or three cystic spaces with the rest a mixture of myxomatous and fibrous tissue (Fig. 11). Cystic kidneys showed varying degrees of hypoplasia. The cysts, which appeared to have arisen from both Bowman's capsule and from the proximal convoluted tubule, were most frequent in the cortex (Fig. 12) and they were so common in some areas that the kidney had a multilocular appearance. There were very obvious changes in the epithelium of the renal pelvis: the transitional epithelium was vacuolated

and, in parts, undergoing degeneration; in some areas there had been proliferation into papilliform ingrowths. Generally throughout the kidneys there was slight interstitial round-cell infiltration. There was an impression that the foetal lobulation was more marked than in the control piglets. Venous congestion was present and there were free red cells in both the cortex and medulla.

Gastro-intestinal tract. There were no consistent changes in the alimentary tract except for an engorgement of the vessels and a very prominent separation and distension of the mesentery by oedema fluid. Fluid was also present beneath the serous coat of the intestine.

Genital tract. The epithelium of the cervix showed slight vacuolation and an increase in thickness. There were no consistent changes in the ovaries and testes.

Endocrine glands. No abnormalities were seen.

Teeth and skeletal system. Unfortunately, the bones and teeth of only one control and one deficient piglet were examined, so that definite conclusions cannot be drawn. No differences were found in the bones. In the teeth of the deficient piglet there was some disorganization of the odontoblasts, and the ameloblasts were difficult to distinguish.

Eyes. All stages of aplasia and hypoplasia (Figs. 13 and 14) were present. When there was no recognizable eyeball, the orbit contained a fibro-cellular and fatty tissue, within which were a few bundles of muscle fibres. In two piglets, this mass of fibro-fatty tissue contained small, irregularly arranged, islands of pigmented epithelial tissue. The upper and lower lids were sometimes continuous and in one case the lids were fused to the cornea. The sclera, when present, was poorly developed and tended to thin out and disappear towards the back of the eye. In many piglets the anterior chamber, the iris, the ciliary body and the posterior chamber could not be identified from a mass of jumbled tissues. The vitreous humour was replaced by fibrous tissue that was myxomatous in places and also vascularized, the network of vessels probably representing the hyaloid artery (Fig. 15). In eyes that were better differentiated, the choroid and the retina were folded. The choroid in particular was ridged and varied in thickness, especially in the region of the ciliary processes, and its epithelial layers showed irregularities. The lens in these cases was poorly developed, the capsule being ill-defined and the so-called lens epithelium apparently absent. The retina showed folding and was separated in places into two not well-defined areas of cells. In some parts of the retina the optic nerve cells were not very numerous and spaces were present in this layer. The rods and cones appeared normal, except in a few areas where they were reduced in number.

Nervous system. Examination of the brain and cord of two piglets failed to reveal any evidence of developmental or other abnormality, such as demyelination. On the other hand, the older pig with posterior paralysis (herd A) showed demyelination in the cord, particularly in the lumbar area, and also slight demyelination in the cerebellar peduncles. There was, however, no histological evidence of an encephalomyelitis.

Skin. There was separation of the collagen fibres with oedema fluid but no other changes, such as hyperkeratosis.

DISCUSSION

In the four herds examined, at least twenty-one litters were a

complete loss or suffered heavy mortality, and a further eleven or more litters contained piglets that lived, but showed a variety of eye defects. Taking all these cases together several general points emerge. In the three herds where the dietary history was known with certainty the basic meal was home-mixed and, as mentioned by Watt and Barlow (1956a), there is always more chance of an unbalanced ration arising under these circumstances than when meal is purchased from a commercial supplier. In the herd where the losses were most serious, vitamin A deficiency was not suspected at once because the basic meal was fortified with a proprietary concentrate and the owner was unaware that this concentrate contained no vitamin A. In a somewhat similar way it is possible to be misled by a superficial account of the management during pregnancy; for in three herds it was stated initially that the affected animals had been at free range, whereas further enquiry established that very little herbage was available at the critical period in each case. Thus the first herd examined (herd A) was of this type and as the early piglets showed severe liver damage, excess pericardial, pleural and peritoneal fluid, together with petechial haemorrhages on the heart, we suspected at the outset that an infective agent was involved. Consequently, transmission experiments were carried out by inoculating a pregnant sow and a litter of new-born pigs with material from the dead piglets. Although, as would now be expected, these inoculations proved harmless, they are quoted to emphasize how the condition may not always be immediately apparent.

Most of the affected litters were from gilts and usually they had been kept housed for most of the time between birth and early pregnancy. From the work of Braude, Kon, Mitchell and Thompson (1951) it appears that older sows can accumulate reserves of vitamin A sufficient to tide them over periods of temporary deficiency, such as when the grazing is poor. However, in contradistinction to the findings of Braude *et al.* (1951), which suggested that in order to produce severely affected piglets the dam had to be brought near to collapse, all the sows and gilts examined in this study were found to be normal in appearance and vigour. This observation could be of importance in field diagnosis. The clinical appearance of the affected piglets was not particularly striking and it is interesting that in all four herds it was the recognition of eye defects which first suggested a known entity. Thus pigs that were viable, yet blind, commanded more attention than those that died shortly after birth; for often in the latter instances the closed lids at death were not so obviously different from those of piglets dead from a variety of other causes. It is also noteworthy that in none of the piglets were there any marked deformities such as the accessory ears, subcutaneous cysts, cleft palate and hare-lip described by Hale (1935).

Herd A appeared to demonstrate the effects of varying degrees

of vitamin deficiency and the findings in all four herds support the conclusion that as the deficiency is ameliorated the changes seen clinically tend to be confined to the eye. Circumstantial evidence from the dietary histories is compatible with the view that a deficiency for only the first month of pregnancy, may still result in blindness (Hale, 1935). Such a view, however, as also the comparable finding in rats (Warkany, 1954), is based on experimental work where an absolute deficiency early in gestation is subsequently corrected and it is more difficult to devise experiments to demonstrate either the effects of a continuing but very low vitamin transfer to the foetus, or a level that is just sufficient only during the first part of gestation. Even so, it is questionable whether information of this type would explain the absence of blindness in the second (experimental) litters from the four sows of herd B. At the first visit to this herd all the survivors from the first three farrowings had eye defects and the fourth gilt, which was seen to farrow, also had piglets with defective eyes. It is known with certainty, therefore, that both the gilts that were left untreated during their second pregnancy had produced blind piglets previously. Furthermore, it was said that the first dead piglets examined by us in this herd came from the same two gilts, and the livers of these piglets were found to be devoid of vitamin A. Having produced piglets that were blind, moribund and depleted of vitamin A, therefore, the two untreated gilts continued on the same ration. Yet, although the second litters died shortly after birth and were still completely deficient of vitamin A, the eyes had developed normally. There is no ready explanation for this unexpected result, but it is fortunate that it occurred because we had come to the view that if piglets could be born viable but blind, then piglets that were moribund at birth could not be expected to have normal eyes. The logical result of this argument would have been to examine the eyes in any litter that died shortly after birth to see whether avitaminosis A should be suspected, but on the basis of the present finding this procedure is probably unsound. It would be interesting to speculate on the mechanisms by which a litter could be moribund at birth but not blind, even though, with the same maternal diet, the litter that immediately preceded it exhibited microphthalmia; but before invoking complicating factors such as the influence of other vitamins and their concentration in the foetus relative to that of vitamin A, it must be recognized that the experimental animals under discussion were not always in our possession. There was a period of 11 days between the last farrowing on the farm and the arrival of the four sows in Cambridge, and although extraordinary precautions were taken to ensure that the untreated sows received no source of vitamin A in Cambridge, it cannot be stated categorically that they could not have gained access to a small amount of carotene during the short unsupervised period.

The main gross pathological changes were the abnormalities

of the eye and the severe oedema. The eye lesions have been described by others, but the marked outpouring of fluid, although seen under comparable conditions in other species, does not appear to have been the subject of previous comment in the pig. In the severely affected animals there was evidence of a very poor circulation and it is probably this and the consequent anoxia which bring about the oedema. The existence of fluid in the body cavities, the pulmonary oedema and the venous congestion of all the organs support the concept of a failing circulation, but no detailed examination was made of the heart and great vessels in order to determine a more precise cause and the primary disturbance might have been elsewhere in the body or even in the placenta. The hearts of many of the piglets were probably dilated, but this is a difficult point to confirm. The presence of tissue anoxia was further substantiated by the histological changes and such anoxia may be significant in the development of the eye lesions.

Two other changes require further comment: those of the kidney and the epithelial surfaces. Congenital kidney lesions are common in piglets and it may be that one of the causes of these abnormalities is a partial maternal deficiency of vitamin A. Gross changes comprising various degrees of aplasia were not uncommon, but microscopic changes were more frequent. The latter were most marked in the renal pelvis and were part of the general pattern of epithelial tissue disorganization. Wolbach and Bessey (1942) epitomized the pathogenesis of the epithelial lesions as 'atrophy of the epithelium concerned, reparative proliferation of basal cells, and growth and differentiation of the new products into a stratified keratinizing epithelium'. In the present study, many of the epithelial surfaces showed changes that ranged from vacuolation to proliferation and accumulation of cells, but nowhere had these changes progressed to the third stage above—the differentiation seen in the adult.

Congenital abnormalities may arise from causes other than vitamin deficiencies, such as by irradiation and mutant genes. Following upon the observations of Young (1952), however, interest has recently centred on the effect of virus infection of the sow during pregnancy as a cause of congenital abnormalities and foetal death. As abnormalities produced by different means may tend to show a common pattern, there is a need for caution in diagnosis. Indeed, the cases of cleft palate, subcutaneous oedema and microphthalmia recently produced in piglets after maternal infection (Harding and Done, 1956) are somewhat reminiscent of the earliest cases ascribed to avitaminosis A. However, given the opportunity to obtain a complete herd history and examine affected piglets, it should be simpler to determine whether the foetus has suffered from a deficiency of vitamin A, than to establish that there has been a maternal infection in early pregnancy, particularly as in the latter instance viruses other than the swine fever virus might be involved.

CONCLUSIONS

Four herds are described in which many piglets died shortly after birth or, if viable, were born blind. At least twenty-one litters suffered heavy mortality and a further eleven or more litters contained piglets that showed a variety of eye defects.

It was concluded that a maternal deficiency of vitamin A was the common cause. In three herds with reliable histories the diet of the affected dams was grossly deficient in vitamin A and in two herds where the analyses were made there was little or no vitamin A in the piglets' livers at birth. In one herd, dosing two pregnant sows with vitamin A palmitate prevented the condition, while two sows left untreated continued to lose their litters.

The dams of affected litters appeared normal and, apart from blindness, the clinical signs in the piglets were not striking. Litters that were moribund at birth were born at full term but showed extreme weakness. Viable piglets showed eye defects, but externally there were no other deformities.

Internally, there were gross abnormalities of the kidney and lung, and one of the most striking features was the generalized oedema. Histologically, there was proliferation and vacuolation, but no metaplasia of epithelial surfaces. The eyes showed a great variety of changes ranging from complete disorganization to near normality.

ACKNOWLEDGMENTS

We are grateful to Miss E. A. Robb and Mr. P. J. D. V. Brett for general laboratory assistance. Most of the vitamin A analyses were carried out in the Dunn Nutritional Laboratory by Miss P. J. Holder and Miss M. C. Anable. The vitamin A palmitate was kindly supplied by Dr. M. D. Wright of Vitamins Limited. The work was assisted financially by a grant to one of us (R.F.W.G.) from the Agricultural Research Council.

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APPENDIX

Herd A

Clinical history. This herd of about twenty Essex sows and a Large White boar had been self-contained, except for boars, for many years. Eight first-cross gilts were retained for breeding in 1955 and they farrowed in 1956, from early February onwards. During February and March six of the gilts, together with four sows, lost their entire litter. It was said that the piglets were listless at birth, with no desire to suckle. During this same period five other sows kept their litters for a week or more, but one of these litters died off from 3 weeks of age, showing signs described as fits with posterior paralysis in some cases. At this stage the problem was referred to us and at the first visit to the farm two survivors from the last-mentioned litter were seen. One of these piglets moved on its abdomen, dragging its hind legs, and it was killed to allow further examination.

The next gilt to farrow was brought to Cambridge on March 29th and the farrowing on April 14th was attended. Although the gilt appeared to be in excellent condition, of the nine pigs born five did not breathe; two of these still-born pigs had grossly distended abdomens. The four live-born pigs were all abnormal in that they were listless, weak and disinterested in suckling. Three were particularly so and these animals did not dry off properly after birth: their skins remained pale and clammy so that skin-like flakes of membrane adhered to them. Their eye-lids remained closed. The remaining live-born pig dried off almost normally and it could just walk. Its eye-lids were open and it lived for between 48 and 60 hours, although it was never seen to suckle well. At about the same time a sow had farrowed ten piglets on the farm. Six of the litter were found dead the morning after farrowing and the remainder, which died during the second night, were received for examination.

The management and feeding can now be described, as the problem was alleviated by a change in the diet. The farm conditions were poor, the sows and gilts running in a small field surrounding a group of sheds that served as a food store and housing. In summer the ground was virtually devoid of grass, and in winter it became a quagmire. The original ration consisted of a mixture of flaked maize, wheat germ, wheatfeed, barley meal, bean meal and minerals. To this was added a proprietary concentrate containing no vitamin A, at the rate of 10 per cent, but this concentrate was replaced on April 30th by one containing 52 million i.u. of vitamin A per ton. Unfortunately, the date of substitution cannot be relied upon precisely. However, although no litters farrowed after this date were lost, there was a period before the advent of completely normal litters, when the piglets were born with a variety of eye defects. Thus a litter of six born on June 2nd included two animals with seemingly normal eyes and four with defects. The next litter of eight, born on June 15th, were normal but for one animal where one eye only was defective. A third litter of ten, born on June 17th, contained three pigs with defective eyes. These abnormalities, in eight piglets altogether, were not examined until July 6th, when it was seen that they were mainly in the sclera and pupil so that the eyeball appeared to be largely white, with only a small pigmented area usually displaced dorsally. Alternatively, the eyeball was all coloured and covered with a grey film. In two piglets there was a unilateral, severe entropion.

One of the original eight gilts, which had lost all its first litter, was served on February 28th and before receiving vitamin A in its ration it was moved to Cambridge on April 17th, it then being 7 weeks in-pig. Thereafter it was fed at 7 lb. per day a meal containing about 4¼ million i.u. of vitamin A per ton until it farrowed on June 21st. In general, the litter of nine live pigs (two more were still-born) lacked vitality, but most of them dried off well and kept suckling. Only one pig showed no interest in suckling and this animal moved about on its abdomen, constantly burrowing into the straw in the manner of the fourth litter in herd B. The whole litter was blind, exhibiting either severe entropion (Fig. 2) or a bulging of the eyeball behind closed lids. The eyelids could usually be opened only with difficulty and in some cases the cornea was hyperaemic or coated with a bluish-grey film. Three pigs were soon overlain and two more were killed on the second day for vitamin A analyses. Their livers contained between 4 and 5 i.u. of vitamin A per g. One more pig was found dead at 5 weeks and hence three pigs were weaned. Two of these animals were kept until they were 8 months old, when they were slaughtered and examined. The orbits of both pigs contained a fibro-fatty tissue within which was pigmented epithelium and cartilaginous tissue (Fig. 4).

Herd B

Clinical history. Four breeding gilts, retained for breeding from two foundation sows, had been kept indoors since birth without any access to green food. The basic meal was ground on the farm after mixing two sacks of barley with four sacks of oats, one sack of wheat and one sack of maple peas. This mixture was then fed as two parts

of the diet with one part of middlings. In June gilt 1 farrowed ten very weak pigs and nine soon died. It was stated that all the litter had either bulging eyes or severe entropion, and the one survivor seen at 3 weeks of age showed bilateral entropion. Gilt 2 farrowed nine pigs the day after gilt 1. Eight survived, although all but two showed eye abnormalities as in herd A, with the added feature in some cases of a bladder-like structure in place of a normal eyeball. Gilt 3 farrowed twelve weak pigs on the same day as gilt 2; only five survived and all these had abnormal eyes as before. Three of the dead pigs from gilts 1 and 3 (not individually identifiable) were received for examination; they were blind and no vitamin A could be detected in their livers. Gilt 4 pigged early in July and the farrowing on the farm was attended. Thirteen pigs were born and all were very weak. Clinically, their behaviour was identical with that of the first litter farrowed in Cambridge from herd A: they moved listlessly on their abdomens, burrowing under the straw and scattering to the corners of the box away from the sow. All were blind; some showed entropion and one piglet possessed a fibro-fatty structure similar to that seen in the two mature pigs from herd A. By 12 hours after farrowing only five pigs remained alive; these were killed and in each the liver contained no appreciable amount of vitamin A.

At this visit to the farm the four sows were bled for serum and the vitamin A levels were 44 (sow 1), 52 (sow 2), 90 (sow 3) and 25 (sow 4) i.u. per 100 ml.

The four sows were then moved to Cambridge for a controlled experiment in which numbers 2 and 4 received vitamin A palmitate (in arachis oil) as a dietary supplement. All four animals were fed the same basic ration as before, it being ground on the farm and sent to Cambridge. Dosing commenced on July 26th and continued twice-weekly until farrowing. The oil was freshly mixed in a little meal on the day of dosing and then fed from a hand-bowl. Approximately 200,000 i.u. of vitamin A was given to the two treated animals by August 8th and thereafter the standard weekly dose was approximately 80,000 i.u. until the end of gestation. No bedding was available until October, when wood chips and a little straw were provided. None was mated until the treated group had had time to re-establish their vitamin A reserves. There was little difficulty in getting the sows in-pig; sows 4 and 2 conceived to their first service on August 21st and October 10th respectively. Sow 3 did not show signs of oestrus and so she was housed with the boar from September 16th onwards; from the farrowing date she had conceived within a few days of this date. Sow 1 was served on September 22nd but returned to conceive on October 13th.

Sow 4 farrowed ten very robust and normal pigs, one of which was killed for examination before suckling, on December 11th, 1956. The liver vitamin A concentration in this latter piglet was 16 i.u. per g. Sow 2 farrowed fourteen pigs, including two that were still-born, on January 31st, 1957. The litter was completely normal and vigorous, and the liver vitamin A concentration in one piglet that was killed on the second day was 26 i.u. per g. In contrast, the litters of both the untreated sows were grossly abnormal and the neonatal livers contained no vitamin A. Sow 3 farrowed fourteen pigs on January 12th, and it is of interest that with an average weight of nearly 2·5 lb. they were not undersized. Sow 1 farrowed on February 4th. Eight pigs were seen but more could have been born later in the night and then eaten. This litter was individually smaller, the average weight being just under 2 lb. Both litters were clinically similar in that the piglets were either born dead or so weak that they could hardly crawl. The live-born animals lay about on their sides in a hunched posture making weak cries, while they often paddled slowly with their legs. As in other litters they were frequently covered with large flakes of membrane (Fig. 1). There was, however, an important difference from earlier litters; for despite the complete absence of vitamin A in all the neonatal livers examined, in no case was there any abnormality of the eyes. These experimental findings are summarized in Table 1.

TABLE 1

EXPERIMENTAL LITTERS FROM HERD B

Sow	*Remarks*	*Piglets born*	*Vit.A (i.u./g. neonatal liver)*	*Litters*
1	Untreated controls	8 (or more)	Zero	Piglets born dead or moribund
3		14	Zero	
2	Dietary supplement of vitamin A	14	26	Piglets normal and vigorous
4		10	16	

Herd C

Clinical history. This herd of about fifty breeding sows farrowed indoors, going out with their litters 5 days after farrowing and returning indoors when the litters were 4 or 5 weeks old. In general, the in-pig sows ran at pasture until about a week or so before term, but twelve gilts born in August, 1955, were yarded after weaning and kept indoors until they were served. Although born at pasture, it is probable that little grass was by then available and after weaning they were fed a home-mixed ration of barley, oats, middlings, fish meal, soya bean meal and minerals. The animals with which we were later concerned farrowed between July 8th and 30th, 1956, and hence their service dates would have been between about March 16th and April 7th. Shortly after service several of the gilts became very weak on their hind legs, as did the boar that was running with them. One gilt aborted, and this, along with some of the paralysed gilts, was slaughtered. Five survivors were then turned out to pasture and although the date of turning-out is not known exactly, it was thought to be the beginning of May. It is possible, therefore, that gilt 32 was 6 weeks in-pig and gilt 41, the last to farrow, about 4 weeks in-pig at this time. It is also probable that with the abnormally dry spring, little grass may have been available at first. All the gilts remained at grass until a week before farrowing and the farrowing histories can be considered, together with those of the older sows that farrowed concurrently, in tabular form (Table 2).

TABLE 2

FARROWING HISTORIES FROM HERD C

Dam	*Farrowed*	*Boar*	*Litter*
Gilt 32	8.7.56	A	All died during the first week.
Sow 42	9.7.56	A	Nine born alive; none blind.
Sow 49	10.7.56	B	Fifteen born alive and one dead; none blind.
Gilt 33	11.7.56	A	Eight born, seven examined. Six bilateral entropion; one unilateral entropion with exophthalmos other eye.
Sow 39	20.7.56	A	Fifteen born alive; three dead (none blind).
Gilt 35	22.7.56	A	Eight born alive. Four survivors examined: one bilateral exophthalmos; two unilateral exophthalmos with entropion other eye; one bilateral entropion.
Gilt 48	23.7.56	A	Seven born alive. Three survivors seen: all severe entropion.
Sow 5	27.7.56	B	Thirteen born alive; none blind.
Gilt 41	30.7.56	A	Eight born alive. Seven examined: all variety of eye defects but not as severe as above; more like the malformations of the later litters in herd A, i.e. only three showed entropion (unilateral).

The above affected litters were examined for the first time on August 14th. It was stated that the litter that died from gilt 32 was weak and unable to suckle strongly; the pigs that died from gilts 35 and 48 were observed to behave similarly.

No histopathological studies were made in this herd.

Plate 1

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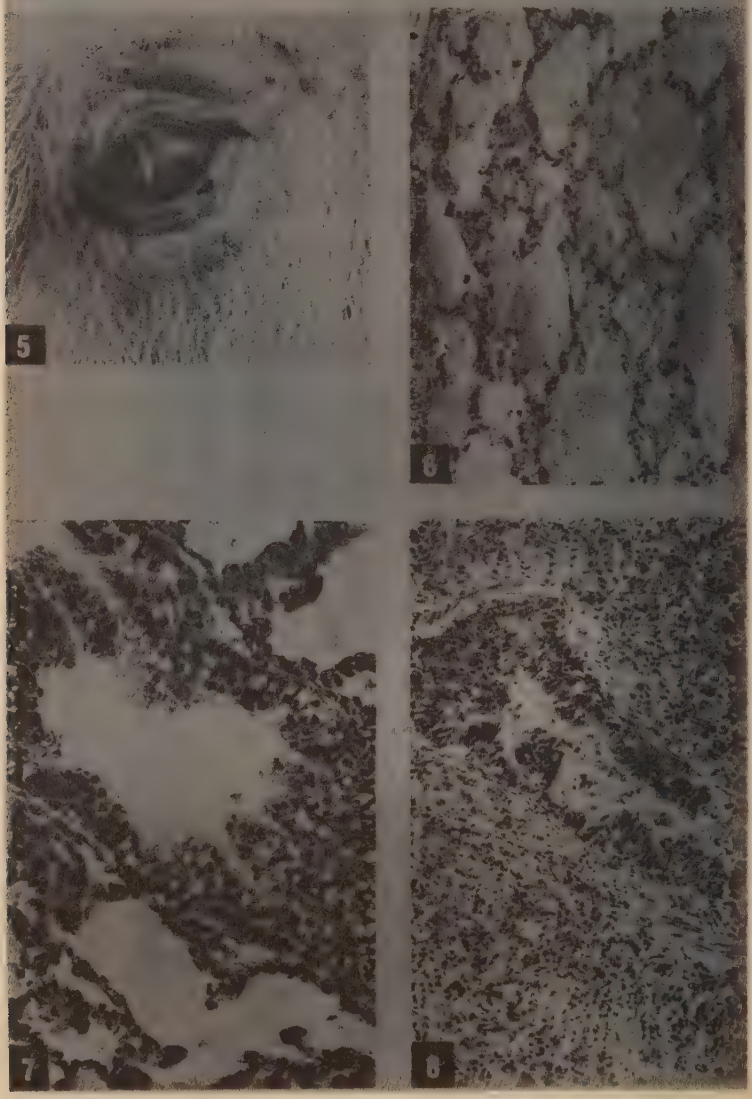
Plate II



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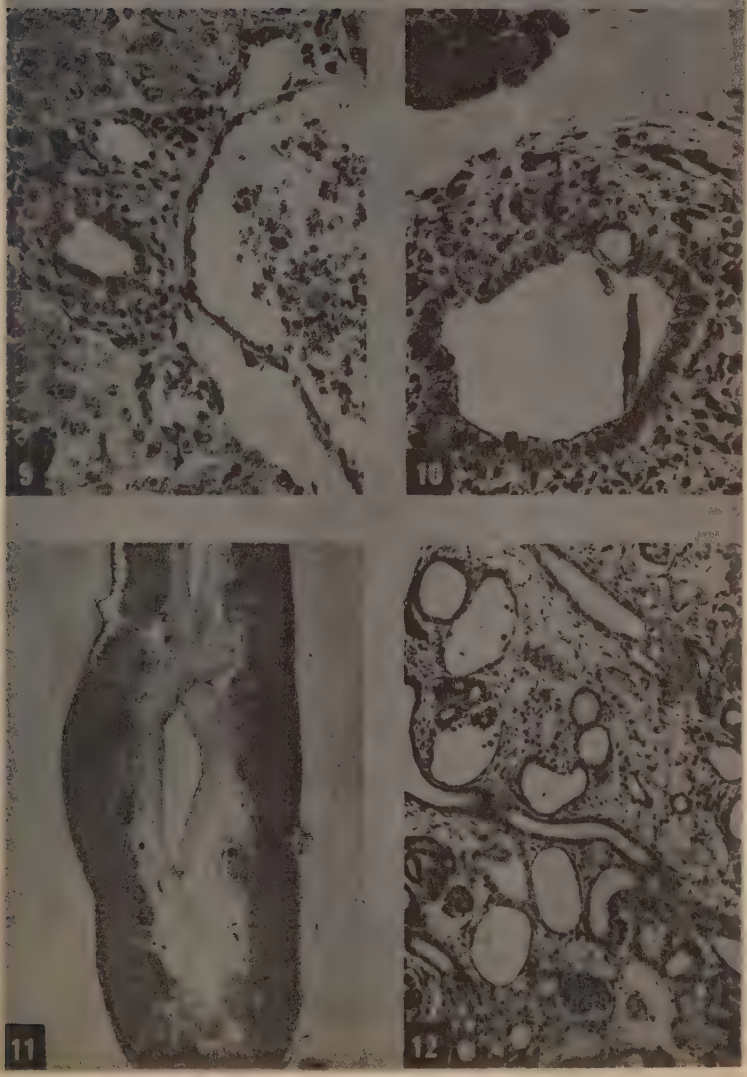

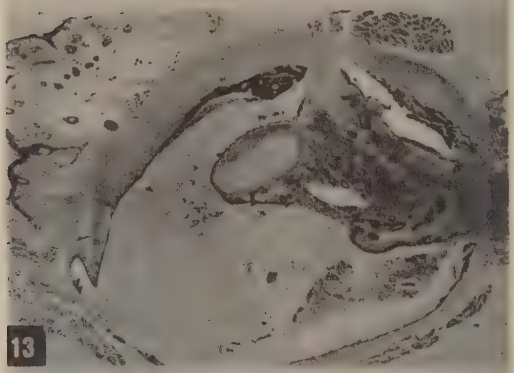
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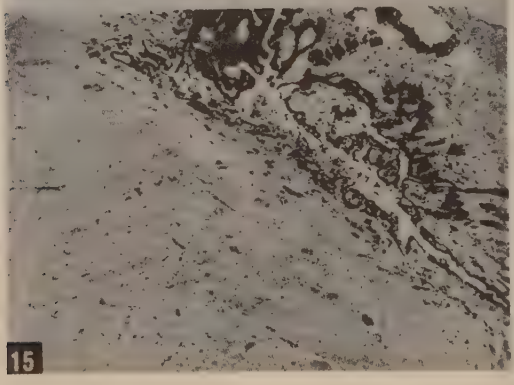
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Herd D

Clinical history. In this case the records were unsatisfactory and more doubt arises. However, the findings warrant a brief report.

Seven gilts were purchased in November, 1955, and kept housed until after they were served between January and March, 1956. Two animals stayed indoors until they farrowed and five went out to grass, most probably in early April. None of the gilts was numbered, but it is known that the one that lost its litter had been housed continuously. Farrowing took place between May 18th and July 10th. One litter of ten pigs was entirely blind and died during the first week. Three other litters were completely blind, mainly with severe entropion, and of a total of twenty-eight pigs, ten died before 4 weeks of age. A further litter of nine pigs was clinically normal but for one blind animal, and the remaining two litters were also normal. The blindness was comparable with that seen before in herds A, B and C. The owner stated that he had been feeding a locally-ground meal throughout pregnancy. Private enquiry at the mill, however, showed that whereas this meal contained 2½ per cent dried grass, none had been supplied to the farm before May, 1956.

Once more, no histopathological observations were made.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Piglets moribund at birth. Dried amniotic flakes and rounded appearance due to anasarca.

Fig. 2. Viable new-born piglet with microphthalmia.

Fig. 3. Eye of piglet 4 weeks old. Abnormal iris, eyelashes stuck together and entropion.

Fig. 4. Vestigial eyes removed from mature pigs: fusion of lids to the eyes. X ⅔.

Fig. 5. Eye showing keratitis in piglet 4 weeks old.

Fig. 6. Section through lung to show pulmonary oedema. X 100.

Fig. 7. Section of bronchial wall to show vacuolation and proliferation of the epithelium. X 100.

Fig. 8. Section of lung to show bronchiole containing desquamated material and mononuclear reaction in the alveoli. X 50.

Fig. 9. Section of liver to show the peri-ductal round-cell infiltration and associated slight oedema. X 100.

Fig. 10. Section through main duct of pancreas to show the peri-ductal round-cell infiltration and oedema. The lining epithelium is vacuolated and becoming multi-layered. X 100.

Fig. 11. Kidney: absence of medulla.

Fig. 12. Cortex of kidney showing cysts. X 100.

Fig. 13. Section through eye to show complete lack of organization. The upper lid is fused to the conjunctiva. The choroid and retina are rudimentary and irregular. There is a fibrous structure from the lens capsule to the optic nerve. × 12.

Fig. 14. Section through eye to show disorganization. The retina is small and folded, as is the choroid. The lens appears to be relatively large and the ciliary processes are vestigial. × 12.

Fig. 15. Section through a vestigial eye to show islets of pigmented, folded epithelium in a stroma of vascular, cellular, fibrous tissue. × 50.

CANINE TOXOPLASMOSIS
THE ISOLATION OF *TOXOPLASMA GONDII* FROM A DOG

By

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INTRODUCTION

Within the last few years increasing attention has been given to the occurrence of *Toxoplasma gondii* infection in man and animals.

Diagnosis of the canine disease has usually been based on histological examination but isolation of the parasite from dogs was achieved by Carini (1911), Carini and Maciel (1913), Nicolau and Kopciowska (1935), Olafson and Monlux (1942) and Yamamoto, Ishida, Fujiwara, Ito and Veda (1955). The investigation of Cole, Docton, Chamberlain, Sanger, Prior and Farrell (1953) is notable in that it also included serological and allergic tests.

Histologically diagnosed cases of canine toxoplasmosis in Great Britain were those of Perdrau and Pugh (1930) Pugh (1948), McIntyre, Trevan and Montgomerie (1948) and the cases described by Lauder, Martin, Gordon, Lawson, Campbell and Watrach (1954) and Campbell, Martin and Gordon (1955) which appeared to have concurrent distemper infection. The only isolations hitherto described in this country were made by Valentine, Lane, Beattie and Beverley (1953), Cathie (1954, 1955), Bain, Bowie, Flint, Beverley and Beattie, (1956) from human cases and by Lainson (1955) who isolated six strains of Toxoplasma from domestic rabbit brain, one strain from Norway rats, two strains from weasels and two strains from ferrets. Other foreign reports of Toxoplasms isolated from man and various animal species are listed in the Bibliography of Toxoplasmosis and *T. gondii* by Eyles and Frenkel (1952, 1954).

The purpose of the present paper is to describe two cases of canine toxoplasmosis from which isolations in mice were attempted, the procedure being successful in one case.

METHODS

Examination of stained films. Impression films prepared from organs at autopsy were dried in air and stained by the Jenner-Giemsa method.

Histopathological methods. Paraffin sections of tissues fixed in formol-sublimate were stained by haemalum-phloxin-tartrazine and by a modification of Wright and Craighead's method which entailed differentiation in tartrazine cellosolve.

Animal inoculations. Suspensions of organs in sterile normal saline were prepared in a mortar and pestle without the aid of an abrasive. Material for passage was treated in all cases with 1,000 units of crystalline sodium penicillin and 400 micrograms of dihydrostreptomycin sulphate per ml. The routes of inoculation are given in the course of the paper.

Complement fixation test. The method of Warren and Russ (1948) as modified by Sabin (1949) was employed. Antigens were prepared from

eggs inoculated on the chorio-allantoic membrane with the canine and RH strains and also from normal chorio-allantoic membranes.

Dye test. The technique described by Sabin, Eichenwald, Feldman and Jacobs, (1952) and modified by Jacobs and Cook (1954) was followed except that a solution of sodium citrate was used as anticoagulant instead of heparin since it was found that citrate afforded a clearer background for reading the test. Peritoneal exudates from mice infected three days previously with the RH and the dog strain were antigens.

Preparation of sera. Two groups of four adult rats were inoculated intraperitoneally with pooled peritoneal exudate from mice infected three days previously with the strains of Toxoplasms. Each rat received approximately 200,000 organisms as estimated by a Neubauer haemocytometer. A third group of four rats was inoculated with a saline suspension of normal mouse spleen having approximately the same nitrogen content as the injected peritoneal exudate when estimated by the micro-kjeldahl technique. After fifty days these rats, together with four normal rats, were anaesthetised by the inhalation of ethyl chloride and bled by cardiac puncture. Sera were separated and pooled for each group and, after the addition of tincture of merthiolate to a final concentration of 1: 10,000 were stored at -20°C. Serum from a dog inoculated with the canine strain was obtained forty days after inoculation. Positive control serum was donated six months after clinical recovery by a student with serologically diagnosed toxoplasmosis.

Sera from three kennel-mates of the dog from which Toxoplasms were isolated were taken a month after the animal's death.

RESULTS

Clinical Data

Case 1. The subject was a two year old male greyhound, one of a group which developed diarrhoea and a fluctuating temperature up to 105°F., over a period of about 14 days. It also showed slight oculo-nasal catarrh and obvious dyspnoea. Until a week before death the appetite was maintained, to be followed by marked anorexia. In spite of treatment with distemper antiserum, penicillin and phthalylsulphathiazole, the animal died.

Case 2. A 3 months old female collie. When first examined the temperature was 103·5°F. Grunting respiration and polypnoea were in evidence. Although the dog was listless and exhibited diarrhoea and intermittent vomition, the appetite was normal. 300,000 units of procaine penicillin were administered. Two days later the temperature was normal and the dog much livelier but the appetite then deteriorated and periods of yelping seemed to indicate pain. Two days later the animal died.

Post-mortem Findings

Both cases showed a similar post-mortem picture. There were several millilitres of sero-haemorrhagic fluid in the pleural and pericardial sacs. Pulmonary congestion, oedema and consolidation had ensued, and numerous foci of necrosis up to 4 mm. in diameter occurred in the parenchyma of the lungs (Fig. 1.). The mediastinal

nodes were inflamed. Multiple necrotic foci were found in the liver and, in case 2, in the spleen and myocardium (Fig. 2). Petechiation of the gastric mucosa and pancreas, and shallow ulceration of the duodenum were features observed only in the first dog.

Microscopical Examination

Toxoplasms were easily demonstrable in impression films of lung, bronchial lymph node, liver and spleen stained by the Jenner-Giemsa method. Sections of lung, bronchial node and liver from both dogs showed lesions similar to those which have been described and illustrated by Campbell *et al.*, (1955), including a number of changes interpreted as evidence of concurrent distemper infection, notably interstitial bronchiolitis with alveolar epithelialisation, cellular degeneration and laminar irregularities in the epidermis of the pads, and phloxinophilic cytoplasmic inclusion bodies widely distributed.

In case 1, there was hyperaemia and petechiation throughout the central nervous system, and early focal gliosis in the cerebral cortex, basal ganglia, thalamus, cerebellum, pons and medulla, some of these lesions being associated with toxoplasms. The parasites were elsewhere concentrated in the lungs, bronchial nodes, liver, gastric wall and spleen.

Case 2 showed foci of necrosis attributable to the activity of toxoplasms in the myocardium, thymus, pancreas, duodenal wall, spleen, mesenteric nodes and adrenals.

Transmission Experiments

Case 1 A 20 per cent suspension of lung, suitably treated with antibiotics, was injected intraperitoneally (I/P) in a dose of 0·5 ml. into each of six mice. Three mice were killed after six days and pooled suspensions of spleen, liver and brain inoculated into six further animals. This procedure was repeated for 20 passages. In none of the mice was it possible to identify *Toxoplasma* in stained smears or sections.

Case 2 Lung, liver, bronchial node and brain tissue were each prepared as described for Case 1, and groups of four to six mice were also inoculated intracerebrally (I/C) with 0·03 ml. of suspensions of lung and of bronchial node. Four days later one mouse of the group inoculated I/P with lung suspension was seen to have a swollen abdomen and was killed. Gross excess of peritoneal fluid was present and stained smears of this material showed the presence of toxoplasms. On the sixth day after inoculation another mouse of this group was killed. The peritoneal fluid was collected aseptically, and found to contain numerous toxoplasms. Peritoneal fluid after treatment with the standard dose of antibiotics was passaged to six further mice (0·25 ml. I/P). Repeated passages were carried out using this technique.

A similar procedure was followed with the groups of mice

TABLE 1

PATHOGENICITY OF CANINE STRAIN

Species	*No.*	*Inoculum*	*Dose*	*Effect*	
Hamster	4	MPF 5+	0·5ml. I/P	3 dead in 5-8 days 1 moribund in 9 days	Marked ascites
Guinea-pigs (500-600g)	4	MPF 6	0·6ml. I/P	2 dead in 6-7 days 2 recovered	1 tested and immune to RH strain
Rats	3	MPF 6	1ml. I/P	Recovered	
Dogs (a) 12 week*	2	MPF 5	1ml. I/P	Recovered	1 tested and immune to RH strain
(b) 10 week*	2	MPF 42	1ml. I/P	1 dead in 6 days 1 moribund in 7 days	Marked ascites
Eggs	—	MPF	Yolk sac or c/a membrane	Embryo dead in 6-9 days	

+ MPF 5: Mouse peritoneal fluid, 5th passage etc.
* Two litter mates of each group received the RH strain concurrently and died in 4-7 days.

inoculated I/P with lymph node, liver and brain, from all of which Toxoplasms were isolated. It is interesting to note that the first mouse to die spontaneously from the first passage belonged to the group of mice inoculated I/P with liver suspension from the dog and this death occurred overnight between the seventh and eighth day after inoculation. Mice from the groups inoculated I/P with material from the lungs or nodes died from the eighth and ninth day onwards, and none from the first group inoculated I/P with brain died at all. Spontaneous death, however, did occur in the latter group from the second passage and from the third passage abundant peritoneal fluid was present. Toxoplasms were also isolated from the mice inoculated intracerebrally with suspensions of lung and of bronchial node.

With progressive intraperitoneal passages through mice the duration of illness gradually diminished in all groups, so that by the fifth passage the mice began to die between the fourth and fifth day until, eventually, the interval seemed fixed at three to five days.

Pathogenicity of the Strain for Other Species

Infective peritoneal fluid from mice was collected aseptically and, after treatment with the standard dose of antibiotics, was used to inoculate hamsters, guinea-pigs, rats and dogs by the intraperitoneal route. The results of these experiments are set out in Table 1. A greater resistance shown by rats to Toxoplasms, as compared with mice, guinea-pigs and hamsters has been described by Jacobs (1953) and appears to be borne out by the present work.

The first experimental infection of two litter-mate pups with the canine strain resulted only in a temporary loss of appetite and slight bloodstaining of the faeces 8 to 10 days after inoculation. A later experiment with pups caused anorexia and fever from the second day, one dog dying in six days and the other being killed in a moribund state a day later. Control groups of two dogs were inoculated with the RH strain in each case with the production of severe, fatal illness. These animals, in common with the second pair of dogs receiving the canine strain, showed at autopsy an abundant peritoneal fluid teeming with Toxoplasms, and sections of liver and brain also contained the parasites.

The canine strain was grown without difficulty in fertile hen eggs following inoculation of the yolk sac or chorio-allantoic membrane and gave rise to lesions on the chorio-allantois resembling those described by Warren and Russ (1948) and McFarlane and Ruchman (1948), with death of the embryo in 6 to 9 days after inoculation.

Toxicity of Peritoneal Fluid

Mouse peritoneal fluid was collected aseptically in 5 ml. amounts and the material centrifuged for 45 minutes at 11,000 r.p.m. in an angle-head centrifuge. Supernatants prepared in this

TABLE 2

COMPARATIVE SEROLOGY OF TOXOPLASMA STRAINS

a). Complement-fixation Test

Complement-fixing Antibody Titre

Antigen	*Rat serum/ canine strain*	*Rat serum/ RH strain*	*Rat serum/ normal*	*Rat serum/ N. mouse spleen*	*Kennel mates serum*	*Dog serum/ positive*
Canine strain	1:8	1:32	0	0	(a) 1:16 (b) 1:20 (c) 1:32	1:128
Human (RH) strain	1:8	1:32	0	0	1:8 1:10 1:16	1:128
Normal C/A membrane	0	0	0	0	0 0 0	0

b) Dye Test

Cytoplasm-modifying Antibody Titre

	Rat serum/canine strain	*Rat serum/RH strain*	*Human serum/ positive*
Canine strain	1:64—1:256	1:256—1:1024	1:1024
Human (RH) strain	1:64—1:256	1:256—1:1024	1:1024

way from mice infected with the RH strain and dog strain proved rapidly fatal when injected in 0·1 ml. amounts intravenously into mice of 18 to 20 g. weight. Peritoneal fluid from a dog infected with RH strain showed similar toxicity for mice. This phenomenon has been described by Weinman (1952) who attributed it to a specific factor which he entitled toxotoxin. The substance has not been demonstrated in peritoneal fluids stimulated by other means, and has no marked antigenicity.

Serological Tests

The strain isolated was tested in parallel with the standard RH strain (Sabin, 1941) using the complement fixation and dye test methods.

Complement-fixation test. Sera from rats inoculated with the RH and canine strains developed complement-fixing antibodies when tested with RH and canine antigens, but did not contain any detectable antibodies against normal chorioallantoic antigen. Normal rat sera and sera from rats immunised with normal mouse spleen were negative against all three antigens. Sera from rats immunised with the RH strain had a complement-fixing titre of 1:32 against both antigens, whereas sera from rats immunised with the dog strain had a titre of only 1:8 against each antigen.

The serum of the dog inoculated with the canine strain had a C.F. titre of 1:128 against both antigens but was negative with the normal chorioallantoic membrane. The sera from the three kennelmates gave titres of 1:8 to 1:16 with the RH antigen, but a two-fold higher titre with the canine antigen (Table 2).

Dye-test. Sera from the rats were tested with antigen from mice inoculated three days previously with RH and dog strains. Titres of 1:256 to 1,024 were demonstrated in the sera of rats inoculated with the RH strain, whereas the titres of the sera of rats inoculated with the canine strain were 1:64 to 1:256, i.e. the sera again gave closely comparable results with the two antigens. Dr. Beverley of Sheffield University kindly performed a dye test with the rat sera and his own RH strain and reported titres of 1/216 for the sera of rats inoculated with the dog strain, and 1/400 for the sera of rats inoculated with RH strain. These results are closely comparable with our own. The positive human serum gave titres of 1 : 1,024 in repeated tests with both antigens.

DISCUSSION

When Toxoplasms cause severe illness in dogs, in cases such as those described in the present paper, a tentative diagnosis may be made on recognising the focal necrotic lesions and by microscopical examination of tissue smears. It is also worth while attempting to isolate the organism. Apart from other factors such as the virulence of the organism, successful isolation may depend upon the time-lag

between the death of the animal and the inoculation of its tissues. Thus in the majority of successful human isolations the parasite was isolated either from biopsy material or from organs a few hours after death. Although Bain *et al.* (1956) obtained Toxoplasms from brain tissue kept at 4°C. for a period of 16 days, and one report by Erichsen and Harboe (1953) describes the successful isolation from the organs of a chicken as late as three days after its death, in general the sooner after death the inoculation of animals is performed the better would seem to be the chance of success (Christiansen and Siim, 1951; Kass, 1955). This view is supported by Yamamoto *et al.* (1955) where, in the case of two kennel mates that died from toxoplasmosis, the parasite was isolated from the dog autopsied almost at once but not from the dog that had died on the previous day. In the cases under description inoculation of infective tissues from the second dog was carried out six hours after death and the failure to isolate the organism from the first case may have been due to the longer lapse of time (12 to 24 hours) between death and subinoculation. In chronic cases, brain appears to offer the best source of isolation (Kass, 1955), but consideration should be given to the suggestion of Frenkel (1949) that experimental infection may not be established because of neutralisation of the relatively few toxoplasms by the low titre of antibody in the affected animal.

According to Frenkel some records of isolations are open to doubt because the experimental animals may have had latent toxoplasmosis. There is need, therefore, to carry out exploratory passages with other animals of the same stock and it is certainly desirable to use more than one species of laboratory animal. In the present work it seems unlikely that the experiments were complicated by a latent infection in view of the ease with which toxoplasms were isolated from Case 2. Of 26 mice inoculated with canine tissue, 12 died spontaneously, and six out of nine killed for passage showed the protozoa. Previous attempts to isolate toxoplasms by repeated passages of material from mouse to mouse had failed and in other experimental work where frequent passages were carried out in mice, latent protozoal infection had not been revealed in the colony.

Regarding the pathogenicity of the isolated toxoplasms, from the results of the first experiment in which only those dogs inoculated with the human RH strain died, the first impression was that the isolated strain was probably less pathogenic. In the second experiment, however, all dogs were affected in the same way suggesting that the isolated strain at least in its 42nd passage through mice was as pathogenic to dogs as the RH.

No difficulty was encountered in preparing antigens and performing the complement-fixation test in this work. Considerable time, however, was spent in becoming familiar with the dye-test technique and overcoming some of the difficulties and inconsistent results with which the dye test confronts all newcomers (Beverley

and Beattie, 1952; Cathie and Dudgeon, 1953; Jacobs and Cook, 1954, Goldman, 1956). With assistance from Professor Beattie and Dr. Beverley we eventually succeeded in carrying out dye-tests.

The results of both serological methods showed that the canine strain behaved almost identically with the RH strain.

It is noteworthy that sera taken from the kennel-mates of the naturally infected dog showed complement-fixation titres of 1:8 to 1:32 a month after its death, suggesting that they also had acquired toxoplasmosis either from the dog that died or from a common source of infection.

CONCLUSIONS

An account is given of attempted isolations of the parasite from two cases of canine toxoplasmosis, one of which was successful.

The pathogenicity of the isolated strain for laboratory animals, including dogs, and for embryonated eggs is described and compared with that of the human RH strain. Infection was associated with the production of a toxic factor in the peritoneal fluid of the mouse and dog. The isolated strain was tested serologically in parallel with the human RH strain and gave results almost identical with it.

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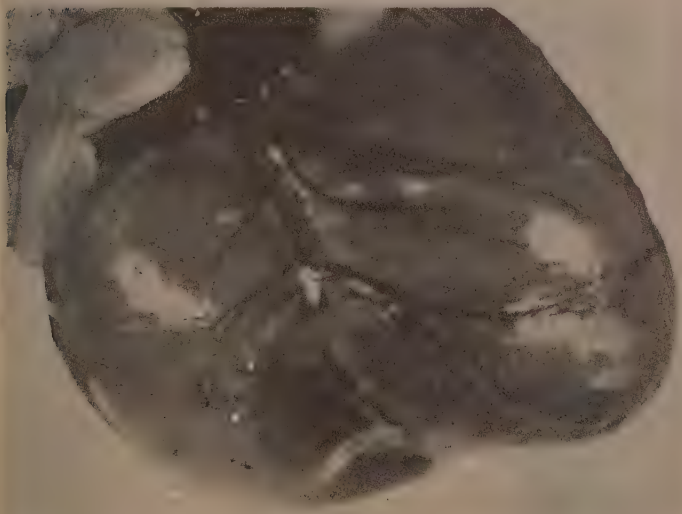
Fig. 1

The lungs are swollen, congested and oedematous. Numerous miliary foci of necrosis are disseminated throughout, and similar lesions occur in the heart. The bronchial nodes are hypertrophic, one of them being visible to the left of the bifurcation of the trachea.

Fig. 2



Detail of the heart showing myocarditis with focal necroses affecting particularly the left ventricular wall.

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THE PATHOLOGY AND BACTERIOLOGY OF BOVINE MASTITIS IN RELATION TO CELL COUNTS

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INTRODUCTION

Savage (1910) observed that in milk from human and bovine mastitis 80 per cent. or more of the cells were polymorphs. Baker and Breed (1920) found that streptococci were present as a rule in samples with very high cell counts. Bourgeois (1927) showed that in samples from ten normal quarters about 50 per cent. of the cells were polymorphs and the remainder were lymphocytes and monocytes, whereas in nine cases of streptococcal and four of staphylococcal mastitis, from 77 to 92 per cent. of the cells were polymorphs. He considered that mastitis was indicated when over 70 per cent. of the cells in the milk were polymorphs. Scholl and Torrey (1931) and Spencer and McNutt (1950) observed variations in severity and type of mastitis in different quarters of a cow and in different parts of a quarter. The former found mastitis in 124 of 165 apparently sterile quarters. Malcolm (1940), Malcolm, King and Campbell (1942), McFarlane (1948), Chu (1949), McFarlane, Blackburn, Malcolm and Wilson (1949), Stableforth (1953) and Hughes (1954) showed that with many inflamed quarters no mastitis organisms can be found in the milk even although the cell count is high. Malcolm *et al.* (1942) also found that there was a higher percentage of polymorphonuclear leucocytes in such milk than in normal milk.

Pattison, Taylor and Holman (1950) found that in artificial infections of the udders of goats with *Str. agalactiae*, there was a predominance of polymorphs at the acute state which lasted for a few days. Thereafter the infection passed to the subacute and chronic stages in which the predominant cells in the milk were epithelial even although *Str. agalactiae* was still present.

In order to obtain additional information on the occurrence of the different cells in milk in cases of mastitis, an investigation was made to determine whether there was any correlation between the total cell content of milk, the proportion of the various types of cells and the presence or absence of mastitis organisms. The bacteriological work was carried out by the staff of the West of Scotland Agricultural College under the direction of Dr. J. F. Malcolm.

METHODS

Ninety-four quarters from 24 abattoir cows in their fifth or later lactation were examined by pathological and bacteriological methods. Milk specimens were cultured on both blood agar and Edwards' crystal violet media. One drop of formalin was then added to each 10 ml. of milk to fix the cells and to prevent the smears from being washed off the

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slides during staining. The smears were prepared and counted by the method described by Blackburn and Macadam (1954).

The skin of the udder was removed without allowing the knife to penetrate the udder tissue and the udder was then frozen. For the bacteriological examination, areas of about three inches in diameter on the lateral surface of the frozen quarter were sterilized by cautery. Udder tissue was then removed by means of a sterile twist drill used in five different directions from the centre of the cauterised area (Blackburn, 1948). Material was taken from three levels. Level 1 included the gland sinus; level 2 was about the middle of the quarter and level 3 was near the centre of the dorsal half of the quarter. Cultures were usually made within an hour after slaughter although some quarters were frozen immediately after slaughter and cultures made a few days later.

Generally four blocks, A, C, D and E, were cut from each of the same three levels as those from which tissue was drilled for bacteriological examination. Block A was from the centre of the level, block C was cut from the anterior angle of a fore quarter or from the posterior angle of a hind quarter, and block D was from the lateral surface of the level. Block E was from the medial surface of a quarter. Techniques of shaping and staining blocks were used as described by McFarlane (1948) and Blackburn (1948). A system of scoring of pathological lesions enables a comparison to be made of quarters containing different lesions (Macadam, 1954). Each inflamed 16th part of a quarter scored as shown in Table 1, the score depending on the extent and type of inflammation.

TABLE 1

SYSTEM OF SCORING OF PATHOLOGICAL LESIONS

Duct system score	*Category of lesion*	*Alveolar system score*
3	Mild acute	3
6	Moderate acute	6
12	Severe acute	12
2	Mild sub-acute	4
4	Moderate sub-acute	8
6	Severe sub-acute	12
1	Mild chronic	2
2	Moderate chronic	4
3	Severe chronic	6

RESULTS

Of the 94 quarters, 14 showed only chronic lesions. Three of the 14 quarters showed a mild chronic mastitis and the cell counts of the milk were 0·75, 1·8 and 8·5 million cells per ml. respectively. One quarter showed a severe chronic inflammation near the gland sinus and this became more moderate towards the dorsal portion of the gland. The watery fluid excreted contained 5·7 million cells per ml., 18 per cent. of which were polymorphs. All these four quarters were almost completely involuted, having been in lactation for nearly a year.

Of the 80 remaining quarters, 20 were affected mainly by chronic mastitis but with some sub-acute inflammation at the lower levels. Ten of the 20 were only slightly affected and gave mean counts of 0·25 million cells per ml., 40 per cent. of these being polymorphs. The low polymorph counts appear to be associated mainly with chronic changes.

In general, high cell counts were associated with acute and sub-acute lesions and polymorph proportions of 51 to 96 per cent. with acute lesions. Three exceptional counts from two cows, which had been in lactation for 6 to 9 months and were giving less than 8 lb. of milk daily at slaughter, showed polymorph percentages of 40, 29 and 17 per cent. respectively in spite of small acute lesions. The extensive desquamation of epithelial cells due to advanced lactation would probably account for the relative depression of the polymorph count despite the presence of small acute lesions. In all three of these quarters, the acute lesions were circumscribed and were about one-sixteenth of the total area of tissue examined. In nine of the 94 quarters at least 90 per cent. of the cells in the milk were polymorphs. Eight of these showed lesions of acute mastitis although in one of these the acute lesions were too small to be included in the score. One cow was slaughtered in advanced involution 19 months after calving and samples from two quarters contained 2,400,000 and 1,200,000 cells per ml., over 90 per cent. of the cells being polymorphs. Minimal discrete lesions of acute inflammation were found in these quarters. Only two of the 94 quarters had negligible pathological changes, and samples taken at the farms contained 80,000 and 190,000 cells with polymorph proportions of 58 and 44 per cent.

TABLE 2

THE RELATIONSHIP BETWEEN CELL COUNT AND TYPE OF PATHOLOGY

No. of quarters	*Mean total cell count*	*Mean % polys.*	*Mean score for pathology*		
			Acute	*Sub-acute*	*Chronic*
	$\times 10^6$				
3	19·0	87	43	107	—
2	7·5	79	123	20	—
4	7·3	82	96	48	8
12	5·5	63	8	37	16
3	5·3	62	33	18	85
2	1·65	84	3	—	—
5	1·35	72	4	—	10
2	1·16	68	11	16	36
1	0·80	70	27	—	20
—					
34					

Table 3 shows the pathology and bacteriology of 67 quarters for which polymorph proportions are available. *Str. dysgalactiae* was not recovered from any of the 94 quarters. The bacteria included

in the mixed group were coagulase-negative staphylococci, *Str. bovis, Str. faecalis, Str. lactis, Esch. coli* and unidentified streptococci. *Corynebacterium pyogenes* and *Str. agalactiae* were as a rule associated with the most extensive pathological changes. In the five quarters infected with *C. pyogenes* about three-quarters of the entire mammary tissue showed severe chronic mastitis and more than half of the remaining tissue, severe acute mastitis. Two of these five quarters were clinically by far the most severely affected by acute mastitis. None of these five quarters was secreting milk at the time of slaughter.

TABLE 3

THE PATHOLOGY AND CELL COUNTS IN RELATION TO BACTERIA FOUND

No. of quarters	*Bacteria found in tissues or in milk*	*Mean total count* $\times 10^6$	*Mean poly.* %	*Mean score for pathology*
7	*Str. agalact.*	8·2	66	162(23C.47S.A.92A.)
15	*Staph. aureus*	6·8	71	82(21C.45S.A.16A.)
1	*Str. uberis*	0·90	96	72(2C. 4S.A.66A.)
6	C.N.S.* & *Str. bovis*	6·4	39	40(19C.19S.A. 2A.)
12	C.N.S.*	2·52	57	35(16C.16S.A. 2A.)
20	None	1·6	58	32(16C.13S.A. 3A.)
4	Mixed	3·08	57	16(7C. 8S.A. 1A.)
2	*Str. bovis*	1·5	69	12(3C. 9S.A.)
67				

* coagulase-negative staphylococci.
C. = chronic; S.A. = subacute; A. = acute.

Although the polymorph proportion of the milk samples varied from 16 to 89 per cent., the milk samples from quarters having *Str. agalactiae, Str. uberis* and *Staph. aureus* in their tissues showed, with one exception, over 50 per cent. polymorphs. Moreover, with quarters infected by the above three organisms, there was a tendency for the lesions to be extensive and acute, whereas with quarters infected with other organisms the lesions were small and acute and there were relatively more sub-acute lesions.

DISCUSSION

The present work shows that the total cell count is as a rule related to the extent of the pathological changes, especially to lesions of acute and sub-acute mastitis. Lesions of mastitis will generally be found in quarters producing milk containing over 200,000 cells per ml. A mean polymorph proportion of 74 per cent. was found in samples from 34 quarters containing lesions of acute mastitis, and a proportion of 90 per cent. was found in the milk from nine quarters, eight of which showed acute mastitis, although in four quarters it was minimal in extent. High polymorph proportions are therefore associated, as a rule, with acute lesions and this is so even although

these lesions are minimal in extent. Quarters showing mainly chronic lesions in which many lobules are involuting, produce milk in which less than 40 per cent. of cells are polymorphs. Combinations of the total and differential counts on samples collected regularly from a quarter can be used to assess the probable pathology. They are also of value in single quarter samples where the stage of lactation is unknown.

In the examination of 94 quarters, *Str. agalactiae, C. pyogenes, Str. uberis* and coagulase-positive *Staph. aureus* were cultured from 23 quarters post mortem and from the milk of seven others prior to slaughter. Other bacteria were cultured from 34 quarters while the remaining 30 quarters were sterile. *C. pyogenes* was associated with the greatest extent of pathological changes, including severe chronic mastitis with complete destruction of secretory tissue. *Str. agalactiae* was associated with severe acute and sub-acute mastitis; in such cases the total cell counts of the milk were as a rule over 3,000,000 and were higher than counts from other affected quarters.

Over 70 per cent. polymorphs, even with a cell count of as low as 250,000 per ml., indicates acute mastitis in progress, although it may be small in extent and of short duration. Two or three days later as the acute inflammation subsides, the cells may be largely epithelial, owing to the fact that large numbers of such cells are desquamated as a result of the closing of acini following inflammation. Where the count rises to about 10,000,000 cells per ml. and about 90 per cent. are polymorphs, mastitis is not only acute and sub-acute but very extensive.

Thirty quarters in which no organisms were found had in general as much affected tissue as those quarters containing coagulase-negative staphylococci, *Str. bovis, Str. faecalis* and *Str. lactis*. Of these 30 quarters, 28 showed inflammation, six having small acute lesions.

CONCLUSIONS

Ninety-four quarters from 24 abattoir cows in their fifth or later lactation were examined. Variations in type and severity of mastitis lesions were found to occur in different parts of the same quarter. Total counts were generally directly related to the extent of pathological changes. Polymorph proportions as a rule exceeded 70 per cent. in milk from acutely inflamed quarters and were below 40 per cent. during involution in the absence of acute mastitis.

The quarters infected with *C. pyogenes* and *Str. agalactiae* showed in general about twice the amount of pathological change present in those infected with *Str. uberis* and coagulase-positive *Staph. aureus*, and about four times the amount shown by sterile quarters and those infected with coagulase-negative *Staph. aureus*.

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FURTHER OBSERVATIONS ON THE MATERNAL TRANSFERENCE OF ANTIBODIES IN THE BOVINE

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INTRODUCTION

In a previous communication under the above title (Blakemore and Garner, 1956) it was reported that when radio-iodinated γ-globulin prepared from the serum of a pregnant cow was re-introduced into the animal intravenously 10 days before parturition, active γ-globulin was rapidly concentrated in the pre-colostrum. Similar experiments have now been carried out using (a) a multiparous cow in the middle part of pregnancy and thus possessing a relatively inactive udder and (b) a heifer due to calve in about 3 weeks into which γ-globulin prepared from another cow was introduced intravenously.

METHODS

γ-Globulin was prepared and iodinated and the radioactivity determinations were made as described in the previous communication.

Cow (*Nancy XVII*) (five months pregnant). In this case 2 g. of the animal's own γ-globulin, iodinated using 5 mc. ^{131}I, was injected into the jugular vein and samples of blood (from the opposite jugular) and mammary secretion were taken at suitable intervals. The volume of the combined secretion from the two hind-quarters of the udder used throughout the course of the experiment seldom exceeded 1 ml.

Heifer (*Elm*). This animal received two intravenous injections, with an interval of one week between the doses, of 2 g. of radio-iodinated γ-globulin from another pregnant cow, Poppy.

RESULTS

Results obtained with Nancy XVII are shown in Fig. 1. At no time did the pre-colostral concentration of active γ-globulin reach that of the plasma indicating that, during the early months of pregnancy in the multiparous bovine, active concentration into the mammary secretion does not take place.

In the heifer Elm (Fig. 2), as in the cow Beech reported earlier (Blakemore and Garner, 1956) labelled γ-globulin was rapidly secreted into the pre-colostrum, the maximum concentration being reached about 5 days after intravenous administration. The concentration of activity in pre-colostrum did not exceed four times that in plasma and, during the greater part of the experiment, remained between two and three times that in plasma. The pre-colostral:plasma ratio is therefore considerably less than the

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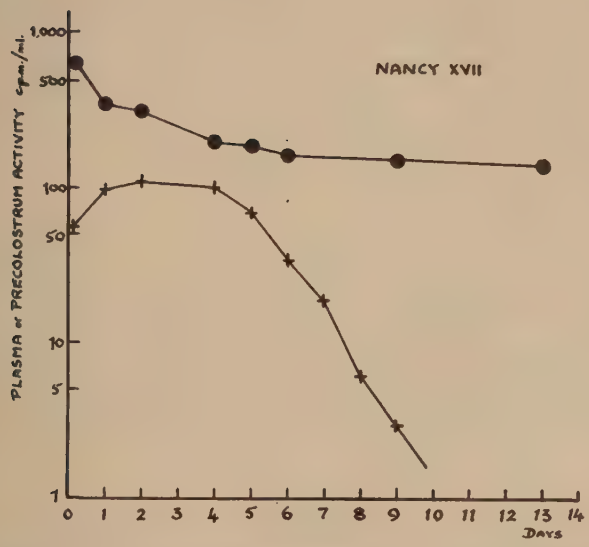
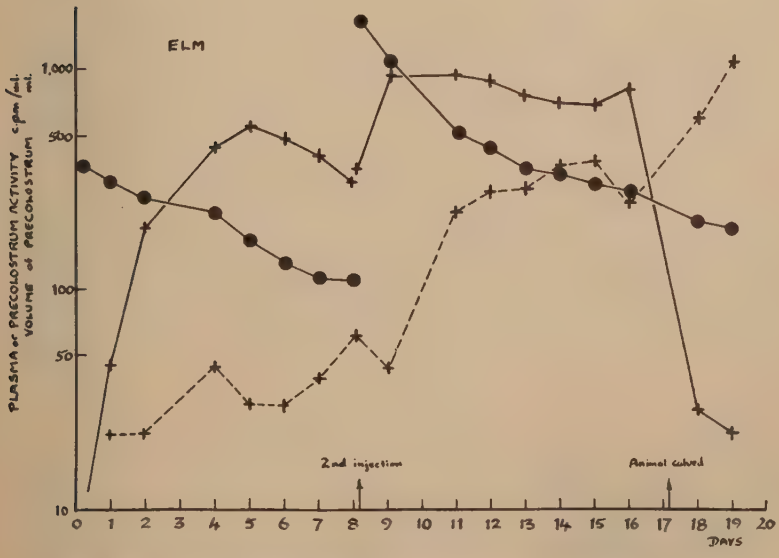
Fig. 1



Cow, Nancy XVII. Graph showing plasma activity (●—●) and pre-colostrum activity (+——+).

Fig. 2



Heifer, Elm. Graph showing plasma activity (●—●), pre-colostrum activity (+——+) and volume of pre-colostrum (+ - - - +).

maximum figure of 13:1 recorded in the cow Beech when the animal's own γ-globulin was labelled and re-introduced. A striking feature is the extremely sudden fall in pre-colostral activity immediately following parturition. This cannot be explained as a dilution effect since the activity of the secretion decreased from 828 c.p.m./ml. on the 16th day to 29 c.p.m./ml. on the 18th day while the volume increased only from 250 to approximately 600 ml. It may indicate a sudden change in the secretory behaviour of the mammary gland at parturition.

CONCLUSION

Homologous bovine γ-globulin labelled with ^{131}I was not concentrated by the mammary gland of a five months pregnant cow.

Labelled γ-globulin prepared from a cow and introduced intravenously into a heifer in the late stages of pregnancy was concentrated to the extent of two to three times in the mammary secretion. Secretion of γ-globulin into the pre-colostrum ceased abruptly at parturition.

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DURATION OF IMMUNITY CONFERRED BY WET LAPINISED RINDERPEST VIRUS VACCINE IN N'DAMA CATTLE IN SIERRA LEONE

By

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INTRODUCTION

The 200,000 cattle of Sierra Leone are all of the N'Dama breed and came originally from the Fouta Djallon area of French Guinea. The first cattle were brought in almost a century ago by nomadic Foulah cattle owners and their numbers have steadily increased as their owners became more settled.

N'Dama cattle have a noteable tolerance to trypanosomiasis but they are very susceptible to rinderpest. Little is known of the history of cattle disease in the early days. It seems that contagious bovine pleuro-pneumonia has always existed, and certainly anthrax also has been recognised, but it is a remarkable fact that until the year 1949 rinderpest was unknown. In this year the first outbreak was recorded, and before the disease was completely eradicated in 1953, mainly by the use of lapinised rinderpest virus, probably half of the cattle population had died. There can be few other areas in tropical Africa where such a large body of susceptible cattle, unprotected in any way, has remained free from rinderpest for such a long time.

It is reasonable to suppose that the 1949 outbreak originated in infected trade cattle entering the country from the north. The system of inspection and movement control of imported cattle is imperfect. It is almost certain that a good many of them escape any form of examination and reach even remote parts of the territory without detection.

Work done in the Gambia had shown that goat-adapted rinderpest virus could cause a heavy mortality in N'Dama cattle. Formalin-inactivated spleen vaccine could be, and for a short time was, used but its limitations are well known. Lapinised virus has proved to be the ideal immunising agent and there can be little doubt that its use has contributed greatly to the control and eradication of rinderpest in Sierra Leone.

METHODS

Production of Lapinised Virus at the Laboratory

Mr. A. Fulton, then Director of the joint Sierra Leone/Gambia Veterinary Service, returned to the Gambia from the October 1948 African Rinderpest Conference in Nairobi with a strain of dried lapinised virus and this was the strain first established in Sierra Leone when the 1949 outbreak of rinderpest occurred. It had been passaged in rabbits

nine times and 1700 cattle had been inoculated when news was received from the Gambia that it was behaving erratically and had been discarded there. It was therefore discontinued here also. The Gambia then received, by air from Cairo, a new strain of dried lapinised virus which probably also came from Kenya in 1948. This strain was received in Sierra Leone after it had been established in the Gambia and was passaged twenty-two times, during which period 8,000 cattle were protected before it was lost because of faulty refrigeration. Rabbits infected with the same strain were again received from the Gambia in September 1950 and the strain has been firmly established here ever since. The virus had been passaged 75 times in the Gambia before it was received and it has since been passaged 141 (141 T) times at Teko. Electricity is not available and the seed virus is kept in kerosene-operated refrigerators. In order to reduce the possibility of losing the strain the virus was at first passaged every ten days, but now only once a month. If the original virus was the Nakamura Strain III (795 passages), the present serial passage would be the 1011th. Over 150,000 cattle have been protected.

The antigenic properties of the virus have been fully maintained and thermal reactions and post-mortem lesions in rabbits appear to be constant.

Rabbits infected intravenously from stock virus are bled 72 hours later. All the heart blood and the spleen is used, but not the mesenteric glands or other glands or organs. The spleen is crushed and added to the blood. Of this mixture 5 ml. is added to 95 ml. of Sorensen's buffer solution and the bovine dose is 1 ml. subcutaneously. One rabbit gives enough virus to immunise between 500 and 600 cattle but it seems probable that, provided the cattle are inoculated quickly enough after the rabbit has been sacrificed, at least double this number could be effectively protected. In Sierra Leone where usually only about 500 cattle are inoculated each day there has been no pressing need to discover the minimum quantity of wet lapinised virus required to immunise and it is known that it is not dangerous to give an overdose.

Rabbits used are of mixed European breeds and the work done in Sierra Leone has confirmed findings in other countries. (Cheng and Fishman, 1948; Brotherston, 1951 a & b; Mornet, Gilbert, Orue and Thiery, 1955; Simpson, 1954; Plowright, 1955).

One interesting point is that it is sometimes possible to evoke a very early thermal response if the serial passage is absolutely direct. On one occasion in Sierra Leone when large numbers of cattle were coming forward, some rabbits inoculated in this way from rabbits used as virus producers showed high temperatures 24 hours later. It was thought that the blood had become infected with an extraneous organism, or that for some unaccountable reason the virus had become exalted in virulence and so all inoculations were immediately suspended. Investigations confirmed that the remarkably quick response was due to the immediate passaging of the fresh virus from rabbit to rabbit and that possibly a larger dose than the normal 1 ml. had been given.

RESULTS

The Reaction of Indigenous Cattle to Lapinised Virus

Thermal response to the virus in susceptible N'Dama cattle has

not diminished and the majority of the cattle will, on the average, show a rise in temperature of 3.8°F on the fifth or sixth day. This feature is so constant (90 per cent) that in the past it was accepted as the criterion of immunisation but, following titration tests with dried virus, it is now known that an absolute immunity can be established even in the absence of a thermal response. Some 50 per cent of cases will show signs of anorexia for two or three days when temperatures are highest, a few will show slight bilateral lachrymation and possibly 20 per cent will have diarrhoea starting on the 9th or 10th day and lasting three or four days. These symptoms are exacerbated in inclement weather and therefore inoculations in Sierra Leone are done in the dry season which is from November to May. Mortality is usually less than 1 per cent.

Duration of Immunity

The cattle used in this experiment were kept isolated and carefully supervised at the Headquarters laboratory. There is no possibility that they came into contact with any infection between immunisation and challenge.

On 6.1.53 twenty-four susceptible N'Dama cattle, all about two years old, were inoculated with 1 ml. of wet lapinised virus, serial passage 82 T. The intention was to challenge these immunised cattle periodically with Kabete 'O' bovine virus until breakdowns occurred. The duration of immunity thus ascertained would have a direct bearing on the intervals between field inoculations. It was known that lapinised virus would protect 'high grade and pure-bred animals of the exotic breeds' for at least two years (Piercy, 1953) and when this experiment was started it was thought that immunity in N'Dama cattle might begin to wane between two and three years. In 1954, however, Datta reported that 'In field tests under Indian conditions an immunity of 41 months was conferred'.

Although 24 cattle were originally immunised only eleven could be brought forward to challenge (Table 1). One animal was destroyed because of a broken femur, one was accidentally shot, one died from snake-bite, one died from causes unknown and the remainder succumbed to a particularly heavy infestation with *Haemonchus contortus* some months after the commencement of the experiment.

It will be noticed that 32 and 47 were challenged with 5 ml. of virulent blood. The explanation of this is that only one ampoule of dried bovine virus of doubtful viability was on hand at the time and so this was injected into two susceptibles in the first place because it was felt that an almost non-viable virus might interfere in some way with the immunity in the test animals if all failed to react and were re-challenged. In fact the two susceptibles reacted typically and pooled fresh blood was used as the challenge material, giving the results shown. All the others were challenged with 0.1g. Kabete 'O' dried bovine virus except the last three (64, 65 and 71)

TABLE 1

CHALLENGE TEST CHART

Cattle No.	*Date inoculated with 1 ml. wet L. R. V.* (82 T)	*Thermal reaction*	*Date of challenge*	*Interval in months*	*Challenge material*	*Reaction*	*Result*	*Reaction of controls*
30	6.1.53	Good*	28.3.53	3	0·1 g. Kabete 'O' bovine virus	Nil.	Immune	Both controls died from rinderpest
74	,,	,,	13.7.53	6	,,	,,	,,	,,
54	,,	,,	4.11.53	10	,,	,,	,,	,,
32	,,	,,	11.10.54	21	5 ml. fresh virulent blood	,,	,,	Both controls died from rinderpest
47	,,	,,	11.10.54	21		,,	,,	
50	,,	,,	10.1.55	24	0·1 g. Kabete 'O'	,,	,,	One control died from rinderpest. The other was slaughtered 'in extremis'
62	,,	,,	27.4.55	28	,,	,,	,,	Both controls died from rinderpest
41	,,	,,	10.8.55	31	,,	,,	,,	Two controls died from rinderpest. The third killed 'in extremis'
64	,,	No reaction	11.2.57	49	0·1 g. Dried Goat virus	,,	,,	All four controls reacted violently. Three died and one recovered
65	,,	Slight reaction	11.2.57	49	,,	,,	,,	
71	,,	Good	11.2.57	49	,,	,,	,,	

* A good thermal reaction indicates a rise of more than 3°F.

which received 0·1g. of dried goat virus. This latter point also requires comment. The whole country had been free from rinderpest for over two years and the use of virulent bovine virus was an unnecessary risk provided another effective challenge material was available.

Following titration tests with dried lapinised virus in cattle it was known that goat-adapted rinderpest virus caused a very severe reaction in N'Dama cattle with a mortality rate of over 70 per cent and that this material therefore constituted a fair challenge. It will be seen from the chart that the four susceptible controls all reacted and that three of them died.

This experiment has proved that lapinised virus is capable of immunising N'Dama cattle against rinderpest for a period of at least 49 months and it seems reasonable to suppose that to all intents and purposes immunity can be considered lifelong.

The Method of Protecting Cattle in the Field

Owing to the extreme fragility of lapinised virus it is the practice in Sierra Leone to take infected rabbits to the site of the cattle inoculations. This arrangement requires that a Veterinary Officer and the Laboratory Superintendent shall be in attendance whenever a rabbit is bled, so that in no case can inoculum be used from rabbits in which the thermal reactions and the post-mortem lesions are not wholly satisfactory. A blood film is always examined for the presence of anthrax before inoculations start and all the work is completed within an hour and a half of the rabbit having been killed. Ice, to cool the diluent, instruments and containers are obtained from a portable refrigerator.

Experience in Sierra Leone tends to show that the danger of using wet lapinised virus does not lie in the possibility of its becoming infected or exalted, but in the failure to immunise because of a very rapid fall in titre under tropical conditions. Field experience and the result of an experiment indicate that an effective immunity is established in N'Dama cattle between 72 and 84 hours after inoculation with lapinised virus.

The present policy is gradually to replace wet lapinised virus by the dried product from Nigeria, but the transporting of large quantities on ice in flasks by air is a most uncertain undertaking and it is necessary to titrate each batch as it arrives. Some consignments have been non-viable or considerably reduced in titre and it seems that wet lapinised virus still has a part to play in rinderpest control in Sierra Leone.

CONCLUSIONS

A brief history of the introduction into Sierra Leone of lapinised virus is given.

Wet lapinised virus is shown to be an excellent immunological agent in N'Dama cattle and the symptoms following its inoculation

are described. Details of an experiment which show that immunity lasts at least 49 months are given.

ACKNOWLEDGMENTS

I should like to acknowledge the help given to me by Mr. N. M. Noble, Senior Veterinary Officer and Mr. S. W. Johnson, Laboratory Superintendent.

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EXPERIMENTAL STREPTOCOCCAL MASTITIS

XII. Further Immunological Studies in the Cow

By

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INTRODUCTION

The objectives of the work recorded in this series of papers on experimental streptococcal mastitis, were to examine the mechanism by which *Str. agalactiae* produces disease of the mammary gland, and to study the behaviour of this organism as an antigen. The pathology and pathogenesis of *Str. agalactiae* mastitis have been described and immunological studies have been made in goats and cows.

In the work already reported on the protective action of *Str. agalactiae* vaccines, the test dose used for production of mastitis was an inoculation of culture directly into the teat canal. With this technique a measurable degree of protection was recorded in vaccinated animals when compared with non-vaccinated controls (Holman, Pattison and Gordon, 1952; Pattison and Holman, 1953; Howell, Smith, Holman and Pattison, 1956). The degree of protection was not great, but a bias in favour of some protection being afforded by the vaccine was found in all experiments. As the method of infection used in these experiments was admittedly artificial, it was considered desirable that an attempt should be made to confirm these observations by testing a *Str. agalactiae* vaccine under conditions more closely similar to those likely to occur in the field.

In this final paper on experimental streptococcal mastitis, therefore, a description is given of an experiment, carried on for 58 weeks, in which 19 vaccinated and 15 non-vaccinated cows were exposed to the risk of infection with *Str. agalactiae* but without inoculation of that organism into the teat canal.

MATERIALS AND METHODS

Housing. The arrangement of the animals within the two isolation buildings is explained in Fig. 1.

Animals. All the cows used were of similar breeding (Ayrshire x Red Poll) and similar age (about two-and-a-half years), and were bred and reared in the Isolation Compound. At the beginning of the experiment some of the animals were dry, but a majority were about mid-way through their first lactation and, as nearly as possible, each vaccinated cow was paired with a non-vaccinated animal of similar milk yield and in the same stage of lactation. As a check for the absence of *Str. agalactiae*, all animals were blood tested before vaccination for agglutinins and hyaluronidase-inhibitors to *Str. agalactiae* strain A100, and all quarters of the animals in milk were examined bacteriologically on three consecutive days. No evidence of *Str. agalactiae* was found in these tests.

Husbandry. All animals received standard maintenance and production rations. Each building was in charge of one man and was equipped with an Alfa-Laval two-bucket milking plant using stretched liners. The milking routine was designed to encourage the spread of organisms from cow to cow. During the first part of the experiment when donor cows were used, these animals were milked first and then without washing or disinfecting the teat clusters, the machines were moved on to the other animals. No disinfectant was used, either on machines or udder cloths, throughout the experiment, but at the end of each milking the buckets were washed and steamed in the usual way.

Period of observation. Considerable variation in calving dates resulted in a corresponding variation in the length of time that individual animals were lactating during the 58-week experimental period. However, all the animals used in the final analysis calved during the experiment and all were kept under observation for at least ten weeks after that calving.

Strain of Str. agalactiae *used.* Strain A100 (Howell, Pattison, Holman and Smith, 1954a) identified serologically (Pattison, Matthews and Howell, 1955) was used throughout the experiment.

Vaccine. The vaccine used was freshly prepared for each inoculation by adding formalin to an 18-hour Todd-Hewitt broth culture of A100 to give a final concentration of 0·35 per cent; a sample plated out 24 hours later showed that the vaccine was non-viable. The vaccination routine was the same as that used by Howell *et al.* (1956). Control cows received no injection. Donor cows experimentally infected with strain A100 were introduced into the herd three weeks after the vaccination routine was completed. Eleven of the vaccinated cows were revaccinated 24 weeks after the first vaccination, during the period when all the cows were dry. This second vaccination consisted of two subcutaneous 10 ml. doses given at weekly intervals.

Methods of spreading infection. In the first part of the experiment, until all cows had finished their first lactation, reliance was placed on natural spread of *Str. agalactiae* from donor cows infected experimentally with strain A100. It is recorded below under "Results" that over a period of twenty weeks no case of *Str. agalactiae* mastitis occurred by spread of infection from the donor cows. After a period of six weeks, during which all cows were dry, it was decided, therefore, to try to increase the chances of spreading infection by regular dipping of the teats of all animals in A100 culture. For this purpose strain A100 was grown overnight in 300 ml. of sterile milk; the culture was then transferred to a polythene beaker into which the teats of all cows were dipped on three consecutive days each week, from the time the first animal calved until ten weeks after the last animal calved (i.e. to the end of the experiment), a period of thirty-two weeks.

Tests for mastitis. The strip-cup was used before the morning milking and abnormalities noted. After the morning milking a clinical examination, including palpation of the udder, was made of the cows in milk. Milk yield was recorded at both morning and afternoon milkings. The strip-cup was helpful in indicating the presence of mastitis, but palpation of the udder was found to be valueless in determining early inflammatory changes.

Quarter samples were taken from every lactating animal once each week before the afternoon milking. A 0·02 ml. drop of milk was spread

over the surface of Bacto-tryptose ox-blood agar and incubated overnight at 37°C. Strain A100 was identified serologically. A neutrophil count was made by the modified Breed smear technique described by Pattison and Holman (1951). The Whiteside test was carried out as described by Howell *et al.* (1954a) and the macroscopic appearance of the milk was graded as described by the same authors.

Criterion of infection with Str. agalactiae *strain A100.* The minimum evidence required before a quarter was accepted as being infected with *Str. agalactiae* was the recovery of strain A100, identified serologically, in two successive weekly milk samples. With one exception, (Table 3, cow 32), the presence of *Str. agalactiae* in a quarter was always accompanied by a rise in neutrophil count above one million per ml. and a Whiteside reading of 2 or over. In a majority of cases neutrophil counts and Whiteside measurements were considerably above these figures.

Criterion of the association of organisms other than Str. agalactiae *with mastitis.* Before an organism other than *Str. agalactiae* was accepted as being associated with mastitis, it had to be isolated from at least two successive weekly quarter samples that had a neutrophil count of at least one million per ml. and a Whiteside measurement of not less than 2.

RESULTS

General

Antigenicity of the vaccine. Agglutination tests carried out on the serum of all cows before the first vaccination and five days after the final vaccination, showed a rise in titre in all vaccinated animals, as recorded in Table 1. The smallest rise was from 1:2 to 1:4 (No. 19) and the largest 1:1 to 1:128 (No. 37). By contrast, the titre of only six of 21 non-vaccinated animals was higher at the second test than at the first and the greatest rise was from 1:1 to 1:4 (No. 36). The antigenic action of this vaccination routine, as judged by the agglutination test, was therefore similar to that observed by Howell *et al.* (1956).

Correlation between tests for mastitis. Previous work reported in this series of papers indicated a correlation between the neutrophil count and the Whiteside test. As indicated in Table 2, this correlation was again shown to hold good and evidence is also given that the macroscopic appearance of the milk may be a useful guide to the degree of mastitis. The figures in this table also show that although *Str. agalactiae* in milk may be associated with obvious evidence of mastitis, it may also be present in milk of normal appearance, negative Whiteside measurement and having a neutrophil count below one million per ml.

Animals discarded during the experimental period. The experiment was started with 22 vaccinated and 21 non-vaccinated animals, housed as shown in Fig. 1. For reasons not connected with mastitis, e.g. late calving, full observations could not be made on nine animals. These cows were omitted from the final analysis which was therefore based on 19 vaccinated and 15 non-vaccinated animals.

Differences in Reaction between Vaccinated and Non-vaccinated Animals

For convenience in analysing differences between vaccinated and non-vaccinated animals, the experimental period may be divided into (a) the twenty weeks at the beginning of the experiment during which donor cows were used to spread *Str. agalactiae* and at the end of which all the animals were dry after their first lactation, (b) the six weeks during which all cows were dry, (c) the 32 week period extending from the first calving after this dry period to the end of the experiment, during which the teats of all animals were dipped three times each week in A100 culture.

TABLE 1

RESULTS OF AGGLUTINATION TESTS

Before and after vaccination

Vaccinated			*Non-Vaccinated*		
	Titre of A100 agglutinins			*Titre of A100 agglutinins*	
Cow No.	*Before vaccination*	*5 days after last vaccination*	*Cow No.*	*Before vaccination*	*5 days after last vaccination*
1	0	4	2	2	4
3	0	32	4	1	0
5	1	16	6	1	0
7	1	4	8	2	2
9	0	32	10	2	0
11	1	32	12	4	4
13	0	64	14	1	0
15	1	64	16	1	0
17	0	64	18	1	0
19	2	4	20	0	0
21	1	32	22	0	0
23	0	4	24	0	0
25	1	4	26	1	1
27	1	4	28	1	2
29	1	32	30	0	1
31	0	16	32	1	2
33	2	16	34	1	0
35	0	16	36	1	4
37	1	128	38	1	0
39	1	4	40	0	0
41	1	32	42	0	2
43	0	4			

Period during which donor cows were used. No cases of *Str. agalactiae* mastitis in either vaccinated or non-vaccinated animals occurred during this period. Teat-swabbing was carried out twice during this time, at approximately two months and four months after the donor cows were first introduced into the buildings. In building 1 *Str. agalactiae* was found on the teats of only one animal at the first swabbing, but in Building 2 the organism was well distributed and occurred on the teats of 12 of the 21 animals. At the second swabbing *Str. agalactiae* was not found on any animal in Building 1 and on only one animal in Building 2.

TABLE 2

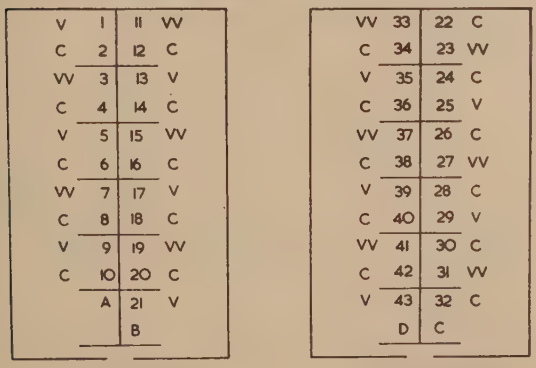
CORRELATION BETWEEN TESTS FOR MASTITIS

Figures for milk samples from cows that became infected with *Str. agalactiae*

Neutrophil Count/ml	*No. of observations*	*Whiteside test*					*Milk appearance*				
		0	1	2	3	4	0	1	2	3	4
Over 10 millions	34	0	0	3	15	16	0	6	13	10	5
6–10 millions	17	0	0	6	9	2	1	7	5	4	0
2–5 millions	43	1	7	18	14	5	6	21	11	6	0
1 million	15	2	3	9	1	0	4	10	1	0	0
Under 1 million	48	12	22	12	2	0	28	19	1	0	0

Fig. 1

THE ARRANGEMENT OF EXPERIMENTAL COWS



KEY: 18 - COW NUMBER 18
C - NON-VACCINATED
V - VACCINATED
VV - TWICE VACCINATED
A, B, C, D } DONOR COWS

NOTE: 5½ Months of this system resulted in no spread of A100.

The donor cows were then dispensed with and the teats of all cows were dipped in A100 culture three times weekly after morning milking.

Period of teat-dipping. During this time infections with *Str. agalactiae* occurred as follows:—

	Vaccinated	*Non-vaccinated*
Total number of cows	19	15
Number of cows infected ..	2	7
Number of quarters infected ..	2	13

The important figures are those for the number of cows infected, and for the four-fold table derived from them: binomial tables (Mainland, Herrera and Sutcliffe, 1956) give a single-sided significance of $p = 0.0234$, or the ordinary double-sided significance of $p = 0.0468$. If one tests the figures for quarters rather than cows, this provides 74 healthy quarters and 2 infected quarters for vaccinated cows, and 47 and 13 for control cows, and Chi squared is about 10·5, giving a probability of 1 in 500. In our view, however, to treat the four quarters of the same cow as different individuals is quite indefensible, particularly as earlier work in this laboratory has shown that the resistance of an animal to streptococcal mastitis is associated, at least to some degree, with the presence of antibodies in the circulating blood.

The duration and severity of the mastitis associated with *Str. agalactiae* strain A100 are shown in Table 3, from which it will be noted that although fewer cows and fewer quarters of vaccinated than of non-vaccinated cows were affected, the degree of mastitis in individual quarters was similar in both groups.

TABLE 3

DURATION AND SEVERITY OF MASTITIS IN COWS WHICH BECAME INFECTED WITH A100

Vaccinated or Control	*Cow No.*	*Quarter*	*Duration of mastitis; weeks after infection**	*Number of weeks in which:—*	
				Neutrophil count exceeded 1 million/ml.	*A100 count exceeded 1000/ml.*
V	1	RF	19+	13	19
V	27	LF	26	7	26
C	2	RF	25+	23	25
C	4	LH	2	0	2
	,,	RH	17	14	16
C	18	LH	16	14	12
	,,	RF	11+	10	8
	,,	RH	3	2	2
C	24	LF	2	1	2
	,,	RF	11	2	11
C	26	LH	17+	16	17
	,,	RH	18	15	18
C	32	RF	7	0	0
C	38	LF	12+	7	12
	,,	LH	5	4	4

* Plus sign indicates that A100 was present at the last sample taken.

As noted in "Materials and Methods," 11 of the vaccinated cows were revaccinated 24 weeks after the first vaccination. This was done because it was considered possible that immunity due to the first vaccination might be waning and it was anticipated that the experiment would continue for at least six more months. It might then have been possible to demonstrate a difference in resistance between cows vaccinated once and those vaccinated twice. However, the result obtained was that of the two vaccinated cows that became infected, one had been vaccinated once and the other twice.

All the 15 quarter infections with *Str. agalactiae* occurred within seven weeks after calving. The distribution of new infections throughout the 58-week experimental period was as follows:—

First lactation	*Second lactation*										
	Dipping of teats into										
Donor cows	infected milk										
	Weeks after calving										Total
	1	2	3	4	5	6	7	8	9	10	
Nil	6	1	2	1	2	1	2	0	0	0	15

These figures cannot necessarily be taken to prove that the cows were particularly susceptible to infection during the first few weeks after calving, because the infection techniques were different in the first and second lactations.

Milk yield. Milk yields of all animals were recorded twice daily throughout the experiment, but extensive attempts to demonstrate a difference in milk yield directly attributable to *Str. agalactiae* infection between the vaccinated animals as a group and the non-vaccinated animals were unsuccessful. Any loss in milk due to *Str. agalactiae* infection was masked by individual variation among animals in the two groups and by variations in yield caused by mastitis due to organisms other than *Str. agalactiae*. This difficulty in expressing in figures the loss of milk caused by *Str. agalactiae* infection was encountered previously by Howell *et al.* (1956), but it does not necessarily mean that this loss is negligible. Indeed, Hale, Plastridge and Williams (1956), by examining a large number of herds over a long period, have shown it to be of considerable importance. Under the conditions of our experiment, however, it was not possible to distinguish between variation in milk yield due to *Str. agalactiae* infection and variation due to other causes.

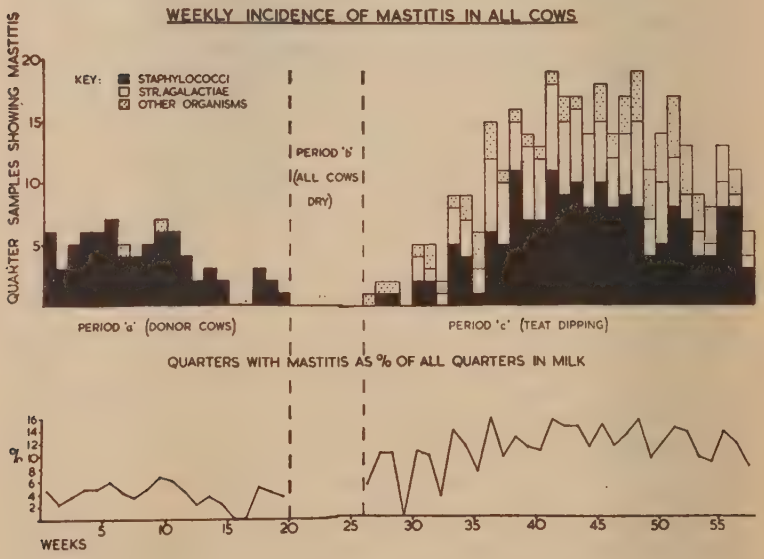
Mastitis due to Organisms other than Str. agalactiae

During the preliminary milk sampling before the experiment began, it was noted that several animals were carrying staphylococci and that *Str. uberis* was present in a few quarters. It was anticipated, therefore, that mastitis due to those organisms might occur during the experiment.

The criterion for diagnosing mastitis due to organisms other than *Str. agalactiae* has been given under "Materials and Methods" as the isolation of the organism from at least two successive weekly

milk samples, together with a neutrophil count of at least one million per ml. and a Whiteside reading of two or over. On this basis mastitis due to organisms other than *Str. agalactiae* was, with two brief exceptions shown in Fig. 2, present in one or more animals throughout the experiment. The weekly incidence of this type of mastitis is shown in Fig. 2, and the number of quarters with mastitis as a percentage of all quarters in milk is also given; this latter measurement averaged 3·8 per cent during period (a), when no *Str. agalactiae* was present, but in period (c), when *Str. agalactiae* cases began to appear, the figure averaged 10·9 per cent. Staphylococci were prominent throughout the experiment. They were frequently associated with mastitis of all clinical types except the peracute gangrenous form and were also often recovered in large numbers from otherwise normal milk samples. The mastitis associated with "other organisms" referred to in Fig. 2 consisted of mild, chronic mastitis from which *Str. uberis* was recovered, usually in association with staphylococci, and two cases of severe, purulent mastitis following accidental injury, from which *C. pyogenes* was recovered.

Fig. 2



DISCUSSION

In his survey of the literature on bovine mastitis to the end of 1935, Munch-Petersen (1938) reviewed the large amount of work done on vaccination. This work consisted almost entirely of field

trials in which many kinds of vaccine were used, very few of which were tested for antigenicity before inoculation. The activity of the vaccines was tested by haphazard exposure to field infection and in a majority of cases no attempt was made to relate the organism, or organisms, in the vaccine to the organisms causing mastitis after vaccination. The absence of control animals was a serious criticism of much of this work. The results of these observations were conflicting, but the general impression was unfavourable to vaccination.

Very little has been published on the use of vaccines in streptococcal bovine mastitis since 1938, probably because research interest has been focussed on therapy with the sulphonamides and antibiotics. The work in this laboratory on experimental streptococcal mastitis that has been reported in a series of publications, of which this is the final paper, was started in 1946 before antibiotics came into general use, and was aimed at obtaining a fuller understanding of the manner in which *Str. agalactiae* causes disease; the investigation has included immunological studies and a reconsideration of the possibility of protective vaccination.

In order to reduce expense and so that relatively large numbers of experimental animals could be used, much of the earlier work was done in the goat when it had been shown that that animal was readily infected with *Str. agalactiae* and that the disease so produced was pathologically indistinguishable from that occurring naturally in the cow (Pattison, 1951). In the meantime mouse-protection and agglutination tests had been developed for the detection of circulating antibodies produced by *Str. agalactiae* (Pattison, Taylor and Holman, 1950, Holman *et al.*, 1952), and at a somewhat later date a suitable serological method for identifying the various serological types of *Str. agalactiae* was worked out (Pattison, Matthews and Howell, 1955). Finally, observations were made on experimental infection of the cow (Howell *et al.*, 1954 a and b). The results of these various investigations were then applied to the problem of measuring the effects of vaccination in the cow, because (a) the serological type of *Str. agalactiae* in the vaccine could be identified with certainty (b) the antigenic activity of the vaccine could be measured (c) the disease process in the cow was understood (d) the organism used to produce the disease could be certainly identified, both when first introduced into the udder and when subsequently re-isolated from the milk. Using this information, a controlled experiment was then carried out on vaccinated and non-vaccinated cows artificially exposed to a test dose placed directly into the teat canal (Howell *et al.*, 1956), and it was found that a significant difference could be shown in various measurements in favour of the vaccinated group.

The experiment described in this paper was planned as a final observation on the protective action of a *Str. agalactiae* vaccine under conditions similar to those that might be expected to occur in the

field. The result of this experiment has given a minimum level of mathematical significance in favour of the vaccinated cows.

When the results of this and earlier experiments with goats and cows are considered as a whole, there can be no doubt that a protective action against *Str. agalactiae* infection of the mammary gland attributable to vaccination with the same strain of organism has been clearly demonstrated. However, it is necessary to emphasize that the degree of protection in any individual experiment has always been small and has been more easily expressed as a bias in favour of the vaccinated animals than as a clearly defined measurement. In these circumstances it is difficult to express an opinion on the practical value of vaccination against *Str. agalactiae* mastitis. The degree of protection would probably be small, but all our experimental data indicate that some protection would certainly be produced. We find it impossible to predict whether the degree of protection thus produced would, from a practical point of view, be great enough to warrant the labour involved in carrying out the vaccination. It is, of course, possible that some more efficient form of vaccine might be produced and an increase in immunising potency thus obtained might make vaccination a practical proposition.

In conclusion, it may be said that because even streptococci, which as a group are well recognized as being poor antigens, have here been shown to have some small beneficial immunising activity, further immunological studies with other organisms that are associated with bovine mastitis would seem to be worthwhile.

CONCLUSIONS

An attempt was made to test a *Str. agalactiae* vaccine under "field conditions" by exposing 19 vaccinated and 15 non-vaccinated cows for 58 weeks to the risk of infection with the same strain of *Str. agalactiae* as was used in the vaccine.

The methods of spreading *Str. agalactiae* through the herd were (a) for the first 20 weeks, until the end of the first lactations, by using experimentally infected donor cows (b) for 32 weeks, after the dry periods, by dipping the teats of all cows three times each week in a culture of *Str. agalactiae*.

Str. agalactiae infections occurred in two vaccinated cows (two quarters) and in seven non-vaccinated cows (13 quarters). This result shows a minimal level of statistical significance in favour of the vaccinated cows.

The practical value of *Str. agalactiae* vaccination is discussed on the basis of the results in this and earlier experiments and the conclusion is reached that the measurable protection given by the method of *Str. agalactiae* vaccination used was too small to be likely to play a significant role in controlling *Str. agalactiae* mastitis in the field.

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